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(54) Title: COMPOUNDS FOR TARGETING ENDOTHELIAL CELLS, COMPOSITIONS CONTAINING THE SAME AND METHODS FOR THEIR USE

(57) Abstract: The present invention provides compounds for targeting endothelial cells, tumor cells or other cells that express the NP-1 receptor, compositions containing the same and methods for their use. Additionally, the present invention includes diagnostic, therapeutic and radiotherapeutic compositions useful for visualization, therapy or radiotherapy.

COMPOUNDS FOR TARGETING ENDOTHELIAL CELLS, COMPOSITIONS CONTAINING THE SAME AND METHODS FOR THEIR USE

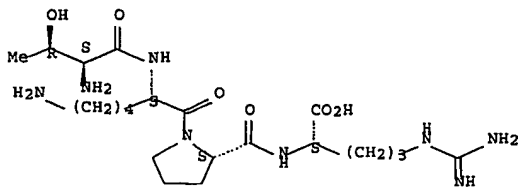
The present application claims benefit of and is a continuation-in-part of U.S. application Serial No. 09/585,364, filed June 2, 2000, the entire contents of which is incorporated herein by reference.

Field of the invention

The present invention relates to compounds useful for targeting endothelial cells or cells that express markers in common with endothelial cells, including certain tumor cells, compositions containing the same, uses thereof and methods for screening them. More particularly the present invention provides novel compounds, and compositions containing the same which may be selectively targeted to endothelial cells, or cells expressing markers in common with endothelial cells accessible to the compositions after parenteral or topical administration, of humans and animals, in vivo and in vitro, the compounds and compositions of the invention may also include a detectable moiety which can be detected by any of the imaging modalities. The compositions of the invention may also include a moiety which is capable of providing a therapeutic or radiotherapeutic effect such as, for example a metal chelating group complexed to a metal ion or a bioactive agent. The compounds of the invention may be used in drug delivery and gene therapy applications. Also provided are methods for using the compounds and compositions of the invention as well as kits containing the same.

Background of the invention

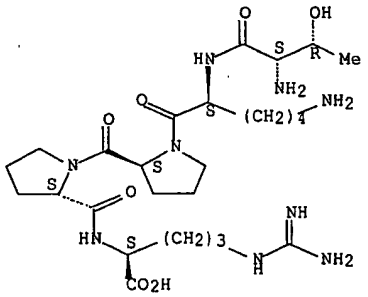
A naturally occurring tetrapeptide TKPR (tuftsin, (SEQ ID NO:1) CAS RN = 9063-57-4), L-threonyl-L-lysyl-L-prolyl- L-Arginine



was discovered to stimulate phagocytosis by binding to receptors expressed on the outer surface of neutrophils and macrophages. Phagocytosis constitutes a major line of defense for a host against bacterial infections. Therefore, as a stimulator of phagocytosis, tuftsin would be expected to be a good peptide for imaging sites of

infectious inflammation. However studies show that tuftsin labelled with a radionuclide metal undesirably accumulates in non-target tissues.

An alternative approach for imaging infection or inflammation based on the use of a radiolabeled tuftsin receptor antagonist has been disclosed by Pollak A., et al, US Patents: 5,480,970, 5,659,041, 5,662,885, 5,569,745 and 5,679,642. These patents disclose the use of Tc-99m chelate conjugates of the tuftsin receptor antagonist (see for a review: Nishioka K. et al., Curr. Med. Chem., 1996, 153-66), , TKPPR (SEQ ID NO:2), (CAS RN = 41961-58-4; or, according to IUPAC nomenclature, L-Arginine, L-threonyl-L-lysyl-L-prolyl-L-prolyl, which has the following structure:



for imaging infection or inflamation. These patents disclose, as chelators, diamidethiols (N_2S_2) and triamidethiols (N_3S). The chelator may beattached to the tuftsin antagonist via a linking group.

Endothelial cells may be defined as an aggregate of cells and/or tissue which may be normal and/or diseased and which may comprise a single layer of flattened transparent endothelial cells that may be joined edge to edge or in an overlapping fashion to form a membrane. Endothelial cells may be found on the free surfaces of the serous membranes, as part of the lining membrane of the heart, blood vessels, and lymphatics, on the surface of the brain and spinal cord, and in the anterior chamber of the eye. Endothelium originates from the embryonic mesoblast and is found associated with heart tissue, including infarcted heart tissue, the cardiovasculature, the peripheral vasculature, such as arteries, veins, and capillaries (the location of which is noted as peripheral to the heart), and the region surrounding atherosclerotic plaques. Additionally, cells that express markers in common with endothelial cells, especially those in contact with the circulation, may also be considered as important targets of the present invention. For instance, melanoma cells that have been observed forming vascular channels and expressing endothelial cell markers as described in A. J. Maniotis et al. (Am. J. Path., 155, 3, 739-752, 1999 and in Science, 285, 5433, 1475, 1999) may be important targets of diagnosis and/or therapy provided by the present invention.

The use of echocardiography for the diagnosis of cardiovascular diseases has generally been limited to indirect methods that involve the detection and

quantitation of abnormalities in the wall motion of the heart. Echocardiography has also been used in connection with methods for detecting pathologies of the heart to identify cardiac masses, emboli, thrombi, vegetative lesions (endocarditis), myxomas, and other lesions.

Accordingly, there is a need for improved imaging techniques, including improved contrast agents that are capable of providing medically useful images of the vasculature and vascular-related organs. The imaging techniques, as used herein, include X-ray Imaging, Magnetic Resonance Imaging, Light Imaging, Scintigraphy, and Ultrasound Echograpy.

In particular, as regards ultrasound echography (ultrasound), the quality of images produced from ultrasound has significantly improved in recent years. New imaging methods, especially dedicated or related to contrast agents have been developed, such as, Native Tissue Harmonic Imaging, 2nd Harmonic Imaging, Pulse Inversion Imaging, Acoustically Stimulated Emission (ASE) etc. Nevertheless, further improvements are needed, particularly with respect to images involving tissues that are well perfused with a vascular blood supply.

Accordingly, there is a need for improved ultrasound techniques, including improved contrast agents that are capable of providing medically useful images of the vasculature and vascular-related organs.

The compounds of the present invention may also be useful in the field of angiogenesis. One of ordinary skill will appreciate that a supply of blood vessels is required for tumors to grow beyond a few millimeters in diameter and to metastasize, and that the process by which the blood is provided is generally referred to as angiogenesis. In this process, a vascular supply is developed from existing vasculature for the growth, maturation, and maintenance of tissue. Angiogenesis is a complex multistep process ,which involves the endothelial cells of the lumen of blood vessels. Endothelial cells contain all the information necessary to proliferate and migrate to form tubes, branches, and capillary networks.

Targeting angiogenic endothelial cells may be achieved by attaching ligands which will selectively bind to molecules which are upregulated in, on, or near these cells.Such molecules include vascular endothelial growth factor (VEGF) receptors such as Flt-1 (also call VEGFR-1), KDR/ Flk-1 (also called VEGFR-2) and NP-1 (also called NRP-1 or neuropilin–1), the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, matrix metalloproteinases, and certain extracellular matrix proteins and fragments thereof. VEGF receptors such as NP-1 or KDR are especially attractive targets. VEGF regulates embryonic vasculogenesis as well as physiological and tumor angiogenesis. Mature VEGF is a homodimer in which the monomers are linked "head to tail" by disulfide bridges. A number of VEGF isoforms are produced by alternative splicing from a single gene

containing 8 exons. $VEGF_{121}$ and $VEGF_{165}$ (containing 121 and 165 amino acids respectively) are the most abundant isoforms. These two VEGF isoforms differ in biological activity. For example, $VEGF_{165}$ is the stronger endothelial mitogen and binds to heparin, while $VEGF_{121}$ does not.

The VEGF receptor KDR is one of two VEGF receptor tyrosine kinases (the other being Flt-1) associated primarily with endothelial cells. KDR is present in low amounts in normal mature vessels, but is strongly upregulated at sites of angiogenesis, including angiogenesis induced by hypoxia, inflammation, and cancer. The main site of KDR expression is endothelial cells, but hematopoietic stem cells, megakaryocytes, and retinal progenitor cells also reportedly express it. In addition, some tumor cell lines may express KDR as well

NP-1 is a transmembrane glycoprotein expressed in developing nervous, cardiovascular and skeletal systems as well as in adult endothelial cells, tumor cells and a variety of tissues including placenta, heart, lung, liver, kidney, pancreas, bone marrow stromal cells, osteoblasts and keratinocytes. NP-1 was first identified as being involved in neuronal cell guidance and axonal growth. However, more recently NP-1 was identified as also being a receptor for $VEGF_{165}$ (and VEGF-B, VEGF-E). Like KDR, NP-1 is strongly upregulated at sites of angiogenesis. NP-1 is a mediator of angiogenesis, particularly in tumors such as breast and prostate carcinoma and melanoma. Cell Vol. 92; 735-74 (1998) Indeed, unlike KDR, NP-1 is abundantly expressed by tumor cells both in vitro and in vivo. Thus, $VEGF_{165}$ binding to tumor cells is mainly due to NP-1. It has been reported that NP-1 expression in tumors resulted in enlarged tumors associated with substantially increased tumor angiogenesis. Further, it has been suggested that NP-1 retains tumor VEGF and prevents its diffusion out of tumor cells. Miao et al "Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression" FASEB J. Vol14, Dec. 2000.

Thus, molecules specific for VEGF receptors like KDR or, more preferably NP-1, should be valuable in diagnosing, imaging and treating angiogenesis.

Angiogenesis is not only involved in cancer development. Many diseases or conditions affecting different physiological systems include angiogenesis. These include: arthritis and atherosclerotic plaques, which may particularly affect bone and ligaments, diabetic retinopathy, neovascular glaucoma, trachoma and corneal graft neovascularization, which may affect the eye, psoriasis, scleroderma, hemangioma and hypertrophic scarring, which may particularly affect the skin, vascular adhesions and angiofibroma, which may particularly affect the blood system. Therefore, anti-angiogenic factors that work by binding to the afore-mentioned receptors could find a use in the treatment or diagnosis of these diseases and tissues or organs, as well as in cancer therapy and diagnosis.

There is therefore a need for an agent which permits visualization by any of the imaging modalities above cited of endothelial cells, and particularly proliferating and or migrating endothelial cells at sites of angiogenesis. There is a further need for a compound that destroys proliferating endothelial cells at sites of angiogenesis thereby starving the tumor by preventing blood from reaching the tumor or for the treatment of inappropriate angiogenesis in general.

Summary of the Invention

It has now been surprisingly discovered that monomers, multimers or polymers of TKPPR, or analogous peptides, may be used to target endothelial cells, or cells that express markers in common with endothelial cells (including certain tumor cells). Specifically, monomers, multimers or polymers of TKPPR or its analogues have been found to target endothelial and other cells by binding to the VEGF binding receptor NP-1. The present invention provides, therefore, new diagnostic and/or therapeutically active agents and methods of their use. Specifically, compounds of the present invention are diagnostic, therapeutic or radiotherapeutic compositions useful for visualization, therapy or radiotherapy of endothelial cells, tumor cells or other cells that express NP-1, such as certain types of tumor cells. In particular, the compound of the present invention may be used for visualization, therapy or radiotherapy of angiogenic tissues or organs.

These compositions comprise a monomer, multimer or polymer of TKPPR (or an analogue of TKPPR which specifically binds to NP-1, endothelial cells, tumor cells or cells that express NP-1) and a pharmaceutically acceptable carrier. The invention also includes diagnostic, therapeutic or radiotherapeutic compositions in which a monomer, multimer or polymer of TKPPR (or a TKPPR analogue) is conjugated, optionally through a linking group, to a substrate.

These compounds are of general formula (I)

A-L-B (I)

in which

A is a TKPPR monomer, multimer or polymer or a monomer, multimer or polymer of a TKPPR analogue, coupled through one or more of the available positions;

L is a linker;

B is a substrate.

The substrate may include, for example, a lipid, a polymer, a detectable moiety or label (including a moiety detectable by ultrasound, MRI, X-ray,

scintigraphy, etc); a bioactive agent (a compound that is capable of providing a biological effect, including a therapeutic or cytotoxic effect), a drug delivery vehicle or a gene delivery vehicle. Thus the substrate may include, for example a metal chelating group optionally complexed with a metal useful in scintigrphic imaging or radiotherapy, a lipid or polymer useful in preparing ultrsound contrast agents (such as, for example gas-filled microbubbles or gas-filled microballoons), a therapeutic or drug, or a delivery vehicle for a drug, therapeutic or genetic material.

Particularly preferred are:

the compounds of general formula (Ia) in which in the general formula (I) B corresponds to

B_1 a lipid able to bind the linker in a covalent or non-covalent manner;

B_2 a non-lipid polymer able to bind the linker in a covalent manner;

B_3 a polymer useful in the preparation of microballoons; or

Bc a chelating group for a metal, which is optionally complexed to a metal.

Even more preferred are compounds of general formula (Ia) in which A is a multimer of TKPPR or a TKPPR analogue, and in especially preferred embodiment A is a tetramer of TKPPR; and B is B_1, B_2, B_3 or Bc as defined above.

The new compounds of the invention may be useful for preparing, in combination with or without a detectable moiety for any of the imaging modalities, novel compositions for imaging and for therapeutic and/or diagnostic applications, where the compounds or compositions of the invention incorporate, for example, a bioactive agent or adetectable moiety, which itself is bioactive (e.g. in Nuclear Medicine).

Brief Description of the Drawings

Figure 1: Activation (phosphorylation) of KDR by VEGF in HUVECs is blocked by a TKPPR tetramer (BRU-326). After the indicated treatments (no VEGF in lane 1 , VEGF in lane 2 and VEGF and BRU-326 in lane 3), KDR was immunoprecipitated and immunoblotted first with anti-phosphotyrosine (top panel) then with anti-KDR (lower panel). Although addition of VEGF alone resulted in a heavily phosphorylated band of KDR on the blot, when the TKPPR tetramer (BRU-326) was added simultaneously with KDR, only a light band of phosphorylated KDR was visible, consistent with about 60% inhibition of KDR activation by BRU-326.

Figure 2: Binding of ^{125}I-VEGF to HUVECs is inhibited by a TKPPR monomer and multimer. Effect of competition with increasing concentrations of unlabeled VEGF (circles), tetrameric TKPPR (BRU-326, squares), and monomeric TKPPR (BRU-95, triangles). Both the TKPPR tetramer and monomeric were able to inhibit binding to ^{125}I-VEGF binding to receptors on HUVECs, although tetrameric TKPPR (BRU-326) was more potent (IC_{50} = 10 μM) than monomeric TKPPR (BRU-95, IC_{50} = 250 μM).

Figure 3: Binding of TKPPR derivatives with NP-1/Fc by Fluorescence Polarization. The indicated Oregon Green-labeled derivatives were incubated with increasing concentrations of NP-1 and polarization was measured. Fluorescently-labeled TKPPR tetramer (BRU-326) bound tightly to NP-1 (K_d = 25-50 nM in different experiments). Binding of TKPPR dimer (BRU-317) was barely detectable by fluorescence polarization (FP). The binding to NP-1 could be competed by unlabeled free TKPPR monomer (IC_{50} = 80 μM) and VEGF (IC_{50} = 200 nM). Binding of OG-TKPPR monomer (BRU-239) to NP-1/Fc was not detectable. Binding of tetrameric TKPPR linked to a different scaffold through the C-terminal amino acid of TKPPR (BRU-346) was also negative. These results are consistent with the radioligand binding data with ^{125}I-VEGF, but also add to them by demonstrating direct binding of the TKPPR tetramer (BRU-326) to NP-1/Fc.

Figure 4: Binding of TKPPR derivatives with KDR-Fc by FP. OG-labeled TKPPR monomer (BRU-239) and tetramer (BRU-326) were incubated with increasing concentrations of KDR-Fc and polarization was measured. . Neither the fluorescently-labeled TKPPR tetramer (BRU-326) nor the fluorescently –labeled monomer (BRU-239) bound to KDR-Fc.

Detailed Description of the Invention

It has now been surprisingly discovered that TKPPR and its analogues (as well as monomers, multimers and polymers of TKPPR and its analogues) bind to the VEGF binding receptor NP-1 on endothelial cells. Further, these monomers, multimers or polymers of TKPPR and its analogues are able to compete with VEGF in binding to endothelial cells. As the binding of VEGF to endothelial cells is necessary for angiogenesis, compounds of the invention and, in particular, compounds comprising a TKPPR tetramer, inhibit VEGF-induced angiogenesis.Thus, the compounds and compositions of the present invention can be useful for

therapeutic applications without requiring the incorporation of other bioactive substances. Specifically, it has been discovered that peptides or pharmaceutically acceptable salts of peptides having the formula

(TKPPR)n

where n is 1 to 30, preferably 1 to 10 or more preferably 4-10, and analogues thereof (e.g peptides which specifically bind to NP-1, endothelial cells or cells that express markers in common with endothelial cells with avidity that is equal to or greater than TKPPR) bind to NP-1 and cells expressing NP-1 (such as endothelial cells and tumor cells). The peptides of the invention block the binding of VEGF to VEGF recptors on endothelial and tumor cells. By blocking the binding of VEGF to these cells, they prevent the angiogenic activity VEGF causes in these cells. Thus, these peptides may be used as therapeutics in the treatment of cancers and other diseases associated with inappropriate angiogenesis. In a preferred embodiment the peptide is multimer of TKPPR or a TKPPR analogue. In a particularly preferred embodiment, the peptide is a TKPPR tetramer.

The peptides of the inventions may be used in a variety of therapeutic and pharmaceutical applications relating to cancers and other diseases associated with inappropriate angiogenesis (i.e. arthritis and atherosclerotic plaques, which may particularly affect bone and ligaments, diabetic retinopathy, neovascular glaucoma, trachoma and corneal graft neovascularization, which may affect the eye, psoriasis, scleroderma, hemangioma and hypertrophic scarring, which may particularly affect the skin, vascular adhesions and angiofibroma, which may particularly affect the blood system.) The peptides of the invention are generally administered as a pharmaceutical composition comprising compounds of the invention or a physiologically acceptable salt thereof, in association with a pharmaceutically acceptable diluent or carrier. Suitable pharmaceutically acceptable carriers include, but are not limited to sterile water, saline solution, buffered saline (including buffers like phosphate or acetate), alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxynethyl cellulose, polyvinylpyrrolidone etc.

The composition may further comprise conventional excipients: i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g. lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings,

and/or aromatic substances and the like which do not deleteriously react with active compounds. The pharmaceutical composition may be prepared by any of the known procedures as described in Remington's Pharmaceutical Sciences, Mack Publishing Co. Eaton, PA 16th Ed, 1980.

The pharmaceutical compositions may be in various forms like tablets or solutions and may be administered by various routes including parenterally (including intravenously, intramuscularly, subcutaneously and intraperitoneally) and in certain embodiments, orally or nasally.

For oral administration, particularly suitable are tablets, dragrees or capsules having talc and/or a carbohydrate carrier binder or the like, the carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

It will be appreciated that the actually preferred amount of active compounds used will vary according the specific compound being utilized, the particular composition formulated, the mode of application and the particular site of administration. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art, using conventional dosage determination tests conducted with regard to the foregoing guidelines.

According to the present invention, a "therapeutically effective amount" of a pharmaceutical composition is an amount which is sufficient the desired pharmacological effect.

Generally the dosage required to provide an effective amount of the composition, and which can be adjusted by one of ordinary skill in the art, will vary, depending upon the age, health, physical conditional, sex, weight and extent of disease, of the recipient. Additionally, the dosage may be determined by the frequency of treatment and the nature and scope of the desired effect. Appropriate dosages will be determined by those of ordinary skill in the art, using routine methods. In treating cancer, particularly small cell lung carcinoma (SCLC), cultured cell lines may also be isolated from a patient and tested for dose responsiveness (Trepel et al., *Biochem, Biophys. Res. Commun.* 156:1383 (1988); Mahmoud et al., *Life Sci,* 44:367 (1989)) Typically, the dose range is from 0.001 to[125] 100 mg of active compound per kilogram body weight. Preferably, the range is from 0.01 to 50 mg. of active substance per kilogram body weight. A preferred composition of the invention is for example, one suitable for oral administration in unit dosage form, for example a tablet or capsule which contains from 1microgram to 500 mg, more preferably from 10 to 100 mg, of peptide in each unit dose, such the a daily oral dose

is from 1 nanogram to 50 milligram per kg of body weight, more preferably from 0.1 to 25 mg/kg, is thereby achieved. Another preferable composition is one suitable for parenteral administration which contains from 0.5 to 100 mg of peptide per ml, more preferably from 1 to 10 mg of peptide per ml of solution, such that a daily parenteral dose of from 1 nanogram to 10 mg per kg of body weight, more preferably from 0.1 to 10 mg/kg, is thereby achieved.

A composition of the invention may also contain, in addition to the peptide of the invention, one of more known bioactive (e.g. therapeutic, cytotoxic) agents, iwhich are discussed imore detail infra.

Analogues of TKPPR include molecules that target the NP-1 VEGFbinding receptor with avidity that is greater than or equal to TKPPR, as well as muteins, retropeptides and retro-inverso-peptides of TKPPR . One of ordinary skill will appreciate that these analogues may also contain modifications which include substitutions, and/or deletions and/or additions of one or several amino acids, insofar that these modifications do not negatively alter the biological activity of the peptides described herein.

The above-mentioned substitutions may be carried out by replacing one or more amino acids by their synonymous amino acids. Synonymous amino acids within a group are defined as amino acids that have sufficient physicochemical properties to allow substitution between members of a group in order to preserve the biological function of the molecule. Synonymous amino acids as used herein include synthetic derivatives of these amino acids (such as for example the D-forms of amino acids and other synthetic derivatives), and ,the D-forms of amino acids and other synthetic derivatives), and may include those listed in the following Table. In the chart and throughout this application amino acids are abbreviated interchangeably either by their three letter or single letter abbreviations, which are well known to the skilled artisan. Thus, for example, T or Thr stands for threonine, K or Lys stands for lysine, P or Pro stands for proline and R or Arg stands for arginine.

Amino acids	Synonymous groups
Arg	Arg, His, Lys, Glu, Gln
Pro	Pro, Ala, Thr, Gly, N-methyl Ala, pipecolic acid, azetidine carboxylic acid
Thr	Thr, Pro, 3-hydroxy proline, 4-hydroxy proline, Ser, Ala, Gly, His, Gln
Lys	Lys, ornithine, Arg, 2-amino ethyl –cysteine, Glu, Gln, His

Deletions or insertions of amino acids may also be introduced into the defined sequences provided they do not alter the biological functions of said sequences. Preferentially such insertions or deletions should be limited to 1, 2, 3, 4 or 5 amino

acids and should not remove or physically disturb or displace amino acids which are critical to the functional conformation.

Muteins of the peptides or polypeptides described herein may have a sequence homologous to the sequence disclosed in the present specification in which amino acid substitutions, deletions, or insertions are present at one or more amino acid positions. Muteins may have a biological activity that is at least 40%, preferably at least 50%, more preferably 60-70%, most preferably 80-90% of the peptides described herein.However, they may also have a biological activity greater than the peptides specifically exemplified, and thus do not necessarily have to be identical to the biological function of the exemplified peptides.

Analogues of TKPPR also include peptidomimetics or pseudopeptides incorporating changes to the amide bonds of the peptide backbone, including thioamides, methylene amines, and E-olefins. Also peptides based on the structure of TKPPR or its peptide analogues with amino acids replaced by N-substituted hydrazine carbonyl compounds (also known as aza amino acids) are included in the term analogues as used herein.

In a preferred embodiment of the invention, a TKPPR targeting molecule, A is conjugated to a substrate and optionally a linker to form conjugated to a substrate and optionally a linker to form the compounds of general formula (Ia), where A is the TKPPR targeting molecule, comprising the TKPPR peptide, or its analogues, or a multimer or polymer of TKPPR or its analogues which can be connected to the linker through one or more of the available functional groups. Preferably, the C- and/or N-terminus of the peptide or peptide analogue are selected for coupling to the linker; however coupling of the linker to an internal amino acid or analogue is an optional embodiment of the present invention.

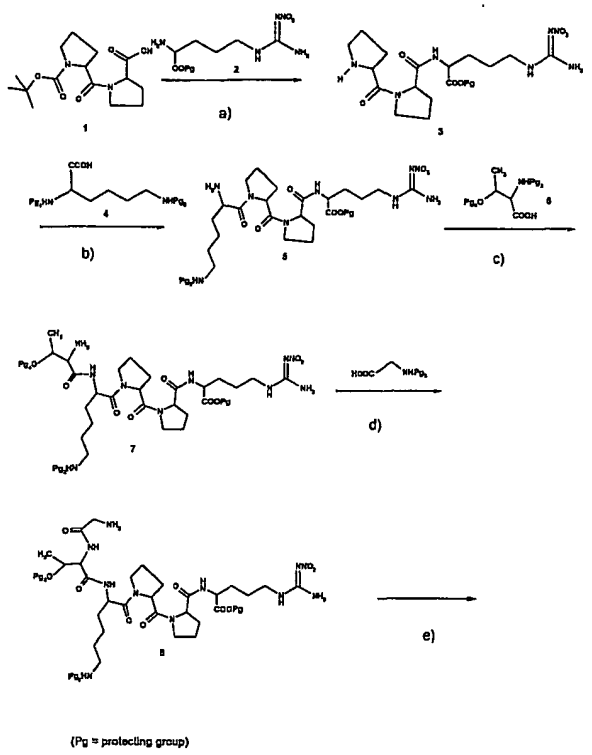
The peptide A is prepared by techniques generally established in the art of peptide synthesis, such as the classical solution approach (Bodansky, M and Bodansky, A The Practice of Peptide Synthesis, Springer Verlag, Berlin, 1984) or the solid-phase approach (Barany, G., Kneib-Cordonier, N., and Mullen, D.G. Solid Phase Peptide Synthesis: A Silver Anniversary Report., Int. J. Pept. Protein Res. 1987 30, 705-739. Fields, G.B. and Noble, R.L. 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Pept. Protein Res. 1990 35, 161-214):

Solid-phase synthesis involves, for example, the stepwise addition of amino acid residues, to the growing peptide chain that is linked to an insoluble matrix or support, such as polystyrene. The C-terminal residue of the peptide, is first anchored (Principles and Practice of Solid Phase Peptide Synthesis ;Fields, G.B., Tian, Z., and Barany, G. "Principles and Practice of Peptide Synthesis" in Grant, G.A. ed. Synthetic

Peptides-A Users Guide Oxford University Press, New York, NY 1992, Chapter 3 pp 104-119) to a commercially available support with its amino group protected with an N-protecting agent such as the t-butyloxycarbonyl group (t-Boc) or a fluorenylmethoxycarbonyl (Fmoc) group and its side-chain, where necessary, protected with a protecting group that is stable to the conditions of the peptide chain extension method (Principles and Practice of Solid Phase Peptide Synthesis Fields, G.B., Tian, Z., and Barany, G. "Principles and Practice of Peptide Synthesis" in Grant, G.A. ed. Synthetic Peptides-A Users Guide Oxford University Press, New York, NY 1992, Chapter 3 pp 81-103). The amino protecting group is removed with suitable deprotecting agents such as TFA in the case of t-Boc or piperidine for Fmoc and the next amino acid residue (with the required N protecting group and its side-chain protecting group, where appropriate) is added with a carbodiimide based coupling agent such as dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) optionally in the presence of an additive such as HOBt (1-hydroxybenzotriazole) or HOAt (1-hydroxy-7-azabenzotriazole), a uronium salt-based coupling agent such as HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate or a related derivative such as HATU [(O-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (Carpino, L.A., El-Faham, A., Minor, C.A. and Albericio, F. J. Chem. Soc. Chem. Commun. 1994 201-203) or a phosphonium salt-based coupling agent such as BOP benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate or related derivatives such as PyBOP ,usually in the presence of 1 to 10 equivalents of a tertiary amine base such as N-methylmorpholine, diisopropylethylamine, triethylamine, 2,4,6-trimethylpyridine (collidine) (Principles and Practice of Solid Phase Peptide Synthesis ;Fields, G.B., Tian, Z., and Barany, G. "Principles and Practice of Peptide Synthesis" in Grant, G.A. ed. Synthetic Peptides-A Users Guide Oxford University Press, New York, NY 1992, Chapter 3 pp 119-125). Upon formation of the peptide bond, the reagents are washed from the support. After addition of the final residue and any other operations, such as removal of the N-terminal Fmoc group (in the case of Fmoc methodology) or addition of any other required moieties to the resin bound peptide by whatever chemical techniques are employed, the peptide is cleaved from the support with a suitable reagent, such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF).

The classical solution approach is illustrated by Scheme 1, which was used in the present invention as an example of the preparation, of the peptide TKPPR or more generally for peptide A.

Scheme 1



The steps a), b), c), and d) are all condensation reactions according to the usual procedures in basic conditions, obtained by adding the appropriate base such as diisopropylethylamine and using a condensing agent, such as DCC, DIC or HATU.

Particularly preferred is HATU ([O-(7-azabenzotriazol-1-yl)1,1,3,3,-tetramethyluronium hexafluorophosphate] , which is particularly effective due to the high reaction rates obtained, the low incidence of side reactions and low racemization at the carbon atom of the incoming amino acid.

The last step e) is the condensation in basic conditions with the suitable linker.

The sterochemistry of the peptide will not be affected by the reactions of the process of Scheme 1, so the absolute configuration of the chiral centers is maintained.

Peptide components are coupled to form a conjugate by reacting the available functional groups present in the molecule with an appropriate functional group of the precursor of the linker L.

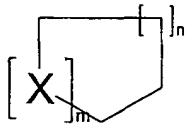
L can be a bond, an alkyl chain C_1-C_{6000}, linear or branched, saturated or unsaturated, optionally interrupted or substituted by one or more groups such as: O, S, NR, OR, SR, COR, COOH, COOR, CONHR, CSNHR, C=O, S=O, $S(=O)_2$, $P=O(O)_2OR$, $P=O(O)_2R$, $P(O)_2(OR)_2$, halogens, or phenyl groups, optionally

substituted by one or more -NHR, -OR, -SR, -COR, -CONHR, -N-C=S, -N-C=O, halogens, in which

R is H or an alkyl group C_1-C_4, linear or branched, optionally substituted by one or more –OH;

such a chain can be interrupted or substituted by one or more cyclic groups C_3-C_9, saturated or unsaturated, optionally interrupted by one or more O, S or NR; by one or more groups such as: -NHR, -OR, -SR, -COR, -CONHR, or a phenyl group optionally substituted by one or more -NHR, -OR, -SR, -COR, -CONHR, -N-C=S, -N-C=O, halogens.

The most preferred, saturated or unsaturated, cyclic groups, according to the previous definition of L, have the following general formula (III)



(III)

in which

n can range from 0 to 4;

m can range from 0 to 2;

X can be NH, NR, O, S, SR, S=O and SO_2;

where R has the same meanings already defined.

In another preferred embodiment the linker L may be an oligopeptide moiety or retropeptide moiety constituted from 1 to 100 natural or synthetic aminoacids. Particularly preferred are glycyl, glutamyl, aspartyl acid, γ-aminobutyryl, trans-4-aminomethyl-cyclohexane carboxyl.

In a preferred embodiment,L precursors are difunctional PEG-(polyethyleneglycol) derivatives.

In a preferred embodiment, L precursors may have the following meaning :

L_1 a linker precursor having on at least two locations of the linker the same electrophile E1 or the same nucleophile Nu1;

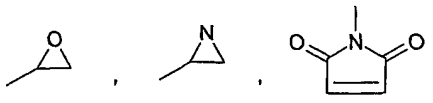
L_2 a linker precursor having an electrophile E1 and on another location of the linker a different electrophile E2;

L_3 a linker precursor having a nucleophile Nu1 and on another location of the linker a different nucleophile Nu2;

L_4 a linker precursor having one end functionalized with an electrophile E1 and the other with a nucleophile Nu1.

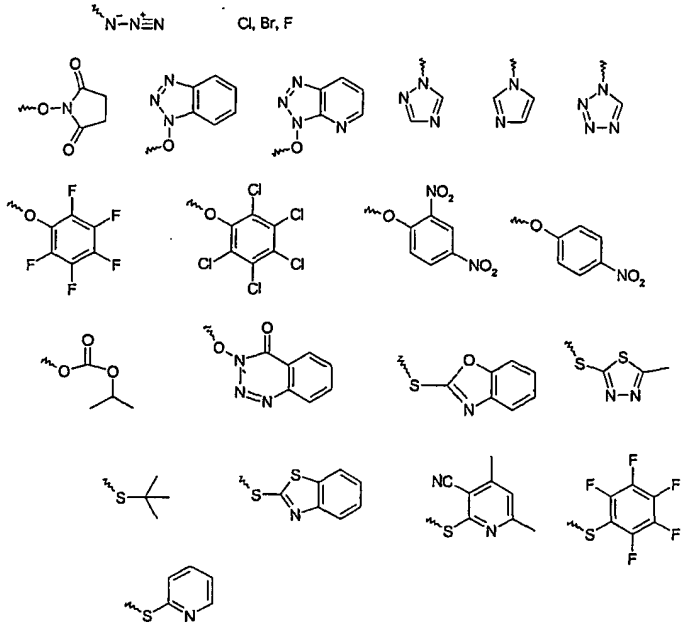
The preferred nucleophiles Nu1/Nu2 of the present invention include -OH, -NH, -NR, -SH, -HN-NH_2, -RN-NH_2, and -RN-NHR', in which R' and R are independently selected from the definitions for R given above, but for R' is not H.

The preferred electrophiles E1/E2 of the present invention include -COOH, -CH=O (aldehyde), -CR=OR' (ketone), -RN-C=S, -RN-C=O, -S-S-2-pyridyl, $-SO_2-Y$, $-CH_2C(=O)Y$,



, wherein

Y can be selected from the following group:



The preferred meanings for L_1 are: suitable derivatives of glutaric acid, succinic acid, malonic acid, oxalic acid, PEG derivatized with two CH_2COOH, wherein the carboxyl functions, prior to the reactions that are performed to form the bonds between the linker L and A or B respectively, have been converted into C(=O)X moieties, either simultaneously or sequentially, employing, if necessary, any intermediate protection or deprotection steps for the carboxyl group that will be used for the second linker bond forming reaction, Y being selected from the set of moieties described in the chart of Y groups shown above. Such procedures and techniques, for sequential deprotection and utilization of similar or identical functional groups, are well known to those of ordinary skill in the art.

The linker L can alternatively be coupled first to the substrate B and then to the peptide.

Particularly preferred are the compounds of general formula (IIb), able to bind the substrate B

A-L (IIb)

prepared according to the general methods above described and where A and L have the meanings discussed herein.

The new compounds of general formula (Ia) may be incorporated in a pharmaceutical composition with different detectable moieties depending on the imaging modality selected.

The incorporation of the targeting moiety may be through a non-covalent association, i.e. a function of a variety of factors, including, for example, the polarity of the involved molecules, the charge (positive or negative), if any, of the involved molecules, the extent of the hydrogen bonding through the molecular network, and the like. Non-covalent bonds are preferably selected from the group consisting of ionic interaction, dipole-dipole interaction, hydrogen bonds, hydrophilic interactions, van der Waal's forces, and any combination thereof. Non-covalent interactions may be employed to bind the compounds of formula (Ia) directly to the surface of various detectable moieties as defined below.

In particular for X-ray Imaging, the new compounds of general formula (I) may be incorporated into X-ray contrast agents, for example, in liposomes encapsulating X-ray iodinated contrast media. Particularly preferred are the liposomes prepared according to the following patents or patent applications: US 5,312,615, US 5,445,810 (WO-A-88/09165) and US 5,393,530 (EP 514523, WO-A-92/10166), US 5,702,722 (WO-A-96/10393), WO-A-96/25955, the entire contents of each of which are hereby incorporated by reference.

For Magnetic Resonance Imaging or for Scintigraphy, the new compounds of general formula (I) may be the targeting moiety for producing new targeted contrast agents, together with the appropriate metal complex, such as those which are known in the fields of radiopharmaceuticals or MRI.

In particular for MRI, the compounds of general formula (I) may be incorporated in lipophilic superparamagnetic contrast agents as those, for example, described in US 5,464,696, US 5,587,199, US 5,910,300, and US 5,545,395 (WO-A-94/04197), the entire contents of each of which are hereby incorporated by reference. Or the new compounds of general formula (I) may be incorporated on the surface of MRI contrast agents based on liposomes or mixed micelles as those described, respectively, in the documents above cited for the X-ray contrast media or in US 5,833,948 (WO97/00087) or in the Serial Application 09/448,289, incorporated herein by reference.

In an analogous way for Scintigraphy, the compounds of general formula (I) may be incorporated in liposomes or mixed micelles comprising suitable complexing agents for radionuclide metals.

Furthermore, in another aspect, the present invention relates to new contrast agents for Scintigraphy or new therapeutic agents for Nuclear Medicine comprising the new compounds of general formula (I), where these compounds include a

suitable complexing agent for a radionuclide and may optionally be incorporated in ultrasound contrast agents, as those defined later, in particular microbubbles or microballoons.

In a preferred embodiment where the compounds of the invention are new agents for scintigraphy or new radiotherapeutic compounds, the substrate comprises a metal chelating group, which is optionally complexed to a metal. These compositions comprise compounds of the formula

A-L-Bc

in which A is a monomer, multimer or polymer of TKPPR or an analogue of TKPPR, L is an optional linker and Bc is a chelating group for a metal. The metal chelating group, Bc, is a molecule that forms a complex with a metal ion that remains stable (i.e. complexed to the metal chelating group) in vivo. Additionally, the metal chelating group is conjugated either directly to the TKPPR targeting moiety or to the targeting moiety via a linker. In a preferred embodiment A is a multimer of TKPPR, such as a TKPPR tetramer.

Metal chelating groups can include monodentate and polydentate chelators [Parker, 1990; Frizberg et al., 1995; Lister-James et al., 1997; Li et al., 1996b; Albert et al., 1991; Pollak et al., 1996; de Jong et al., 1997; Smith et al., 1997]. For example, chelating groups may include tetradentate metal chelators which can be macrocyclic or non macrocyclic, have a combination of four nitrogen and/or sulphur metal-coordinating atoms [Parker et al., 1990; Li et al., 1996b] and are designated as N_4, S_4, N_3S, N_2S_2, NS_3 chelators. A number of suitable multidentate chelators that have been used to conjugate proteins and receptor-avid molecules. [Frizberg et al., 1995; Lister-James et al., 1997; Li et al., 1996b; Albert et al., 1991; Pollak et al., 1996; de Jong et al., 1997]. These multidentate chelators can also incorporate other metal-coordinating atoms such as oxygen and phosphorous in various combinations. The metal binding complexing moiety can also include "3+1" chelators [Seifert et al., 1998]. For radio diagnostic applications, Oxa-Pn AO ligands, are preferably used. These ligands are discussed in US Patent Nos. 6,093,382 and 5,608,110, which are incorporated by reference herein in their entirety.

The metal which is optionally complexed with the chelating group can be any suitable metal chosen for a specific therapeutic or diagnostic use, including paramagnetic metals, lanthanides, auger electron-emitting isotopes, positron-emitting isotopes, transition metals, and α, β or γ emitting isotopes. The type of medically useful metal ion depends on the specific medical application. The compounds of this invention that contain a chelating group may be employed as ligands for the formation of radioactive or non-radioactive metal complexes. Metal complexes may be formed by complexing a ligand with a metal having an atomic number 22-31, 39-

49, 57-71 or 73-82, especially a radioactive metal, preferably under basic conditions. Preferred metal complexes are those containing a radioactive metal such as ^{99m}Tc, ^{67}Ga, ^{68}Ga, ^{111}In, ^{88}Y, ^{90}Y, ^{105}Rh, ^{153}Sm, ^{166}Ho, ^{165}Dy, ^{177}Lu, ^{64}Cu, ^{97}Ru, ^{103}Ru, ^{186}Re, and ^{188}Re.

Radionuclides of the elements Tc and Re are particularly applicable for use in diagnostic imaging and radiotherapy. Other radionuclides with diagnostic or theraupetic applications include, but are not limited to ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{97}Ru, ^{109}Pd, ^{166}Ho,, ^{198}Au, ^{149}Pm, ^{166}Dy, ^{175}Yb, ^{117}Sn, ^{199}Au, ^{203}Pb, ^{211}Pb and ^{212}Bi.

Technetium complexes are particularly useful for radiodiagnostic applications. The technetium employed is preferably one or more of the radionuclides ^{99m} Tc, ^{94m} Tc or ^{96}Tc-. The preferred radioisotope for medical imaging is ^{99m}Tc. Its 140 keV γ-photon is ideal for use with widely available gamma cameras. It has a short (6 hour) half-life, which is desirable when considering patient dosimetry. ^{99m}Tc is readily available at relatively low cost through commercially produced ^{99}Mo/^{99m}Tc generator systems. Preparation of the complexes of this invention where the metal is technetium may be accomplished using technetium in the form of the pertechnetate ion. For ^{99m} Tc, the pertechnetate ion is preferably obtained from commercially available technetium-99m parent-daughter generators; such technetium is in the +7 oxidation state. The generation of the pertechnetate ion using this type of generator is well known in the art, and is described in more detail in U.S. Pat. Nos. 3,369,121 and 3,920,995. These generators may generally be eluted with saline solution, and the pertechnetate ion obtained as the sodium salt. Pertechnetate may also be prepared from cyclotron-produced radioactive technetium using procedures well known in the art.

These metal complexes find utility as diagnostic and/or therapeutic agents. The choice of metal ion will be determined based on the desired therapeutic or diagnostic application. The metal complexes of the present invention may be administered by any appropriate route such as orally, parenterally (for example, intravenously, intraperitoneally, intramuscularly, or subcutaneously), or by any other suitable method. For example, the complexes of this invention may be administered to a subject by bolus or slow infusion intravenous injection. The amount administered may be selected based on the desired use, such as to produce a diagnostic image of an organ or other site of a subject or a desired radiotherapeutic effect, by methods known in the art. Exemplary dosages are those employing about 30-200 mCi rhenium (for radiotherapy) or about 10-60 mCi technetium (for imaging).

An exemplary method for the formation of a metal complex with ligands disclosed herein is where a complex or salt of the desired metal in the desired oxidation state and containing one or more easily displaceable (i.e. labile) ligands (for

example, H_2O, halogen (e.g. Cl), NO_3^-, or sugars) is mixed with ligand(s) at a pH value suitable for forming the desired complex. The labile ligand(s) are displaced from the metal by the ligand(s) of the present invention to form a metal complex.

Illustrative methods are shown as follows:

$$(Met)(Lig_{lab})4 + (Lig_{inv}) \quad (Met)(Lig_{inv}) + 4(Lig_{lab}) \qquad (1)$$

where

Met is a metal in a desired oxidation state;

Lig_{lab} is a labile ligand such as H_2O, Cl^-, Br^-, F^- or NO_3^-; and

Lig_{inv} is a ligand comprising a chelating group, an optional linker and a targeting peptide of the invention (e.g. a monomer, multimer or polyner of TKPPR or a TKPPR analog).

$$(Met)OCl_4^- + (Lig_{inv}) \quad (Met)O(Lig_{inv}) + 4Cl^- \qquad (2)$$

$$(Met)O_2(Lig_{mono})_4 + (Lig_{inv}) \quad (Met)O_2(Lig_{inv}) + 4(Lig_{mono}) \qquad (3)$$

where Lig_{mono} is a monodentate ligand such as pyridine, halide, phosphine or amine.

$$(Met)(Lig_{bi})_2 + (Lig_{inv}) \quad (Met)(Lig_{inv}) + 2(Lig_{bi}) \qquad (4)$$

or

$$(Met)O(Lig_{bi})_2 + (Lig_{inv}) \quad (Met)O(Lig_{inv}) + 2(Lig_{bi}) \qquad (5)$$

where Lig_{bi} is a bidentate ligand such as a sugar, a diol, a bisamine, bipyridine or phosphine, and where, for each equation (1) to (5) above, the appropriate charge balance is employed.

Alternatively, metal complexes may be prepared from a metal in an oxidation state different from that of the desired complex. An exemplary such method is that where either a reducing agent or an oxidizing agent (depending on the oxidation state of the metal used, and the oxidation state of the desired final product) is added to the reaction mixture containing metal to bring the metal to the desired oxidation state. The oxidant or reductant may be used to form an intermediate complex in the desired oxidation state but with labile ligands which are then displaced by a desired chelating ligand of the present invention; or the oxidant or reductant may be added to the reaction mixture containing metal along with the desired ligand to achieve the

change to the desired oxidation state and chelation to the desired metal in a single step.

The formation of the ^{99m}Tc complexes of the invention is acheived by mixing pertechnetate ion in normal saline with the appropriate chelating ligand. An appropriate buffer or physiologically acceptable acid or base may be used to adjust the pH to a range of about 3 to about 9.5, depending on the chelating ligand that is chosen. A source of reducing agent is then added to bring the pertechnetate down to the desired oxidation state for chelation with the ligand. Stannous ion is the preferred reducing agent, and may be introduced in the form of a stannous salt such as stannous chloride, stannous fluoride, stannous tartrate, or stannous citrate. The reaction is preferably run in an aqueous or aqueous/alcohol mixture, at a temperature that may range from room temperature to about 100°C, using a reaction time of about 5 minutes to about 1 hour. The reducing agent should be present at a concentration of 5-50 ug/mL. The ligand should optimally be present in a concentration of 0.1-2 mg/mL.Alternatively, the technetium complexes of this invention can be prepared by ligand exchange. A labile Tc(V) complex can be prepared the reaction of TcO_4^- with a readily exchangable ligand such as the hydroxycarboxylate ligands glucoheptonate, gluconate, citrate, malate, mannitol, tartrate, or aminecarboxylates such as EDTA or DTPA at a pH value that is appropriate for the exchange ligand in question (usually 5-8). A reducing agent such as the stannous salts described above is added, which causes the formation of a labile reduced complex of Tc with the exchange ligand. This reduced Tc complex is then mixed with the desired chelating ligand at an appropriate pH value, and the labile exchange ligand is replaced by the chelating ligand bearing TKPPR, thus forming the desired technetium complexes of this invention.

Rhenium complexes are particularly useful in radiotherapy applications. The rhenium employed is preferably one of the radionuclides Re-186 or Re-188, or a mixture thereof. Preparation of the complexes of the present invention where the metal is rhenium may be accomplished using rhenium starting materials in the +5 or +7 oxidation state. Examples of compounds in which rhenium is in the Re(VII) state are NH_4ReO_4 or $KReO_4$. Re(V) is available as, for example, $[ReOCl_4](NBu_4)$, $[ReOCl_4](AsPh_4)$, $ReOCl_3(PPh_3)_2$ and as $ReO_2(pyridine)_4^+$. (Ph is phenyl; Bu is n-butyl). Other rhenium reagents capable of forming a rhenium complex may also be used.

It is convenient to prepare the complexes of this invention at, or near, the site where they are to be used. A single, or multi-vial kit that contains all of the components needed to prepare the complexes of this invention, other than the Technetium ion, is an integral part of this invention.

A single-vial kit would contain ligand, a source of stannous salt, or other pharmaceutically acceptable reducing agent, and be appropriately buffered with pharmaceutically acceptable acid or base to adjust the pH to a value of about 3 to about 9. It is preferred that the kit contents be in the lyophilized form. Such a single vial kit may optionally contain labile or exchange ligands such as glucoheptonate, gluconate, mannitol, malate, citric or tartaric acid and can also contain reaction modifiers such as diethylenetriamine-pentaacetic acid (DPTA), ethylenediamine tetraacetic acid (EDTA), or α,β,or γ cyclodextrin that serve to improve the radiochemical purity and stability of the final product.

A multi-vial kit could contain, in one vial, the ingredients except pertechnetate that are required to form a labile Tc(V) complex as described above. The quantity and type of ligand, buffer pH and amount and type of reducing agent used would depend highly on the nature of the exchange complex to be formed. The proper conditions are well known to those that are skilled in the art. Pertechnetate is added to this vial, and after waiting an appropriate period of time, the contents of this vial are added to a second vial that contains the ligand, as well as buffers appropriate to adjust the pH to its optimal value. After a reaction time of about 5 to 60 minutes, the complexes of the present invention are formed. It is advantageous that the contents of both vials of this multi-vial kit be lyophilized. As above, reaction modifiers, exchange ligands and stabilizers may be present in either or both vials. In addition, bulking agents such as mannitol, that are designed to aid in the freeze-drying process, may be present.

The amount of radiopharmaceutical administered may be selected based on the desired use, such as to produce a diagnostic image of an organ, by methods known in the art. Doses may range from about 2 to 200 mCi, or as limited by the in vivo dosimetry provided by the radiopharmaceuticals.

Furthermore, in another embodiment of the invention, the compounds of general formula (I) may be incorporated in commercially available microparticles, such as fluorescent microspheres to provide compounds and methods for in vitro and in vivo (only for animals) screening of microbubble or microballoon agents of the present invention. This is because derivatized microspheres display the targeting vector on their surface in a manner similar to that expected for microbubbles and microballoons, hence they can act as a model for the latter two types of entities. One example of such microspheres is Fluospheres® (Molecular Probes Corporation, Eugene, Oregon USA) which possess a carboxylate or amine-modified modified functionality which allows attachment of a peptide via its N-terminus or C-terminus to the bead, using kits provided by the company. The microspheres may be from 0.02 microns to at least 4 microns

in diameter and possess fluorescent dye moieties in a variety of colors (blue, yellow-green, Nile Red, orange, red-orange, Crimson Infra-red, or Far red, for example). The microparticles are stable to physical stress as may be encountered in vivo, particularly in the vasculature, whether they are stationary or in transit throughout the entirety of the circulatory system including the periphery, the cardiovascular system and the entirety of the pulmonary vasculature.

In this case the greater stability of the micoparticles over that of the micobubbles allows more rigorous assays and assay conditions to be used. This is useful because the bubbles are much more fragile than the beads and are much less able to survive the assay procedures whether automated or not. In addition the bubbles float which makes exposure to the substrate on the bottom or sides of the well/assay system difficult. The beads do not float and are robust so are well-suited to the assays. The microparticles or beads may have a detection system. The detection system may use light or radioactivity.

In a preferred embodiment of the present invention, the new compounds of general formula (I) may be useful for preparing new targeted diagnostic and/or therapeutically active agents useful, for ultrasonic echography, by incorporating them in different ways into ultrasound contrast agents. In a further embodiment, the present invention provides ultrasound contrast agents containing a compound and/or composition of the present invention.

An ultrasound contrast agent of the present invention may be in any convenient form, for example, a contrast agent of the present invention may be in the form of a gas-containing or gas-generating formulation and it comprises a plurality of targeting moieties of formula (I) incorporated in the chemical structure.

Gas microbubbles and other gas-containing materials preferably have an initial average size not exceeding 10 μm (e.g. of 7 μm or less) in order to permit their free passage through the pulmonary system following administration, e.g. by intravenous injection.

In particular, the gas containing contrast agents of the present invention may include suspensions of gas filled microbubbles or suspensions of gas filled microballoons, according to the definition given, for example, in EP 554213, and US 5,413,774.

The term "microbubble" specifically designates gas bubbles, in suspension in a liquid, preferably also containing surfactants or tensides to control the surface properties and the stability of the bubbles. Preferably the microbubble suspension comprises a surfactant or a tenside, such as, for example, a polyoxyethylene-polyoxypropylene block copolymer surfactant such as Pluronic® or a polymer

surfactant such as those disclosed in US 5,919,314. More preferably, amphipapathic compounds capable of forming stable films in the presence of water (or an aqueus camer) and gas are used as surfactants in the stabilized microbubbles. Such compounds may include, for example, a film forming lipid or preferably a phospholipid.

The term "microcapsule" or "microballoon" designates preferably air or gas-filled bodies with a material boundary or envelope, i.e. a polymer membrane wall. Both microbubbles and microballoons are useful as ultrasonic contrast agents.

Furthermore, it may be possible to encapsulate a drug in the interior or attach it or incorporate it in the encapsulating walls of the agents of the present invention. Thus the therapeutic compound may be linked to a part of the wall, for example through covalent or ionic bonds, or may be physically mixed into the encapsulating material, particularly if the drug has similar polarity or solubility to the membrane material, so as to prevent it from leaking out of the product before its intended action in the body. The destruction of gas-filled microballoons using external ultrasound is a well -known phenomenon, e.g. as described in WO-A-9325241 or US 5,425,366; the rate of release may be varied depending on the type of therapeutic application by using a specific amount of ultrasound energy from the transducer.

The therapeutic agent may be covalently linked to the encapsulating membrane surface using a suitable linking agent. Thus, for example, one may initially prepare a hydrophobic derivative to which the drug is bonded through a biodegradable or selectively cleavable linker, followed by incorporation of the material in the microballoons. Alternatively, hydrophobic derivatives that do not require processing to liberate an active group may be incorporated directly into the membrane. The active drug may be released by increasing the strength of the ultrasound beam.

In a preferred embodiment, the substrate comprises a lipid, B_1, and the compounds of the invention are incorporated into gas-containing microbubbles. The lipids B_1, are synthetic or naturally-occurring compounds, and are generally amphipathic and biocompatible, comprising a hydrophilic component and a hydrophobic component. The lipids B_1 usable for preparing the gas-containing agents of the present invention include, for example: fatty acids; lysolipids; phospholipids such as: phosphatidylcholine (PC) with both saturated and unsaturated lipids, including phosphatidylcholines such as dioleylphosphatidylcholine; dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine (DLPC); dipalmitoylphosphatidylcholine (DPPC); disteraoylphosphatidylcholine (DSPC); and diarachidonylphosphatidylcholine (DAPC); phosphatidylethanolamines (PE), such as dioleylphosphatidylethanolamine,

dipalmitoylphosphatidylethanolamine (DPPE) and distearoyl-phosphatidylethanolamine (DSPE); phosphatidylserines (PS) such as dipalmitoyl phosphatidylserine (DPPS), distearoylphosphatidylserine (DSPS); phosphatidyl-glycerols (PG), such as dipalmitoylphosphatidylglycerol (DPPG), distearoylphosphatidylglycerol (DSPG); phosphatidylinositol; sphingolipids such as sphingomyelin; glycolipids such as gangliosides GM1 and GM2; glucolipids; sulfatides; glycosphingolipids; phosphatidic acids as dipalmitoylphosphatidic acid (DPPA) and distearoylphosphatidic acid (DSPA); fatty acids such as: palmitic acid; stearic acid; arachidonic acid; oleic acid; lipids bearing polymers, such as chitin, hyaluronic acid, polyvinylpirrolidone or polyethylene glycol (PEG), also referred as "pegylated lipids", with preferred lipids bearing polymers including DPPE-PEG (DPPE-PEG), which refers to the lipid DPPE having a PEG polymer attached thereto, including, for example, DPPE-PEG2000, which refers to DPPE having attached thereto a PEG polymer having a mean average molecular weight of about 2000; lipids bearing sulfonated mono- di-, oligo- or polysaccharides; cholesterol, cholesterol sulfate and cholesterol hemisuccinate; tocopherol hemisuccinate; lipids with ether and ester-linked fatty acids; polymerized lipids (a wide variety of which are well known in the art); diacetyl phosphate; dicetyl phosphate; stearylamine; cardiolipin; phosholipids with short chain fatty acids of about 6 to about 8 carbons in length; synthetic phospholipids with asymmetric acyl chains, such as, for example, one acyl chain of about 6 carbons and another acyl chain of about 12 carbons; ceramides; non-ionic liposomes including niosomes such as polyoxyethylene fatty acid esters, polyoxyethylene fatty alcohols, polyoxyethylene fatty alcohol ethers, polyoxyethylated sorbitan fatty acid esters, glycerol polyethylene glycol ricinoleate, ethoxylated soybean sterols, ethoxylated castor oil, polyoxyethylene-polyoxypropilene polymers, and polyoxyethylene fatty acid stearates; sterol aliphatic acid esters including cholesterol sulfate, cholesterol butyrate, cholesterol iso-butyrate, cholesterol palmitate, cholesterol stearate, lanosterol acetate, ergosterol palmitate, and phytosterol n-butyrate; sterol esters of sugar acids including cholesterol glucoronides, lanosterol glucoronides, 7-dehydrocholesterol glucoronide, ergosterol glucoronide, cholesterol gluconate, lanosterol gluconate, and ergosterol gluconate; esters of sugar acids and alcohols including lauryl glucoronide, stearoyl glucoronide, myristoyl glucoronide, lauryl gluconate, myristoyl gluconate, and stearoyl gluconate; esters of sugars and aliphatic acids including sucrose laurate, fructose laurate, sucrose palmitate, sucrose stearate, glucuronic acid, gluconic acid and polyuronic acid; saponins including sarsasapogenin, smilagenin, hederagenin, oleanolic acid, and digitoxigenin; glycerol dilaurate, glycerol trilaurate, glycerol dipalmitate, glycerol and glycerol esters including glycerol tripalmitate, glycerol distearate, glycerol tristearate,

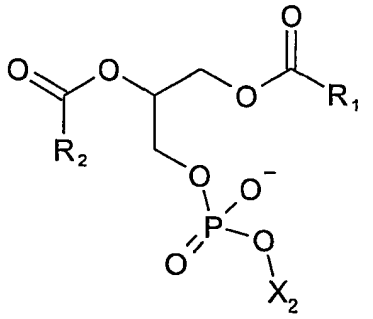
glycerol dimyristate, glycerol trimyristate; long chain alcohols including n-decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol, and n-octadecyl alcohol; 6-(5-cholesten-3β-yloxy)-1-thio-β-D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholesten-3 β-yloxy)hexyl-6-amino-6-deoxy-1-thio- β-D-galactopyranoside; 6-(5-cholesten-3 β-yloxy)hexyl-6-amino-6-deoxyl-1-thio- β-D-mannopyranoside; 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid; N-succinyldioleylphosphatidylethanolamine; 1,2-dioleyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoylglycerophosphoethanolamine and palmitoylhomocysteine, and/or combinations thereof.

In a more preferred embodiment, the present invention refers to new compounds of general formula (IIa),

$$A\text{-}L\text{-}B_{1a} \quad \text{(IIa)}$$

in which

B_{1a} corresponds to a phospholipid moiety of general formula (II),



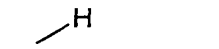
M (II)

and

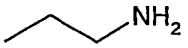
M is an alkaline or alkaline- earth metal cation

R_1 and R_2 independently, correspond to linear long chain C_{12}-C_{20}, saturated or unsaturated, optionally interrupted by C=O, or O

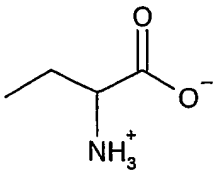
X_2 can be selected from a group consisting of the following meanings



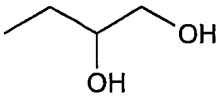
phosphatidic acid



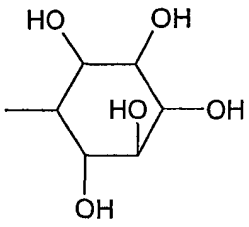
ethanolamine



serine



glycerol



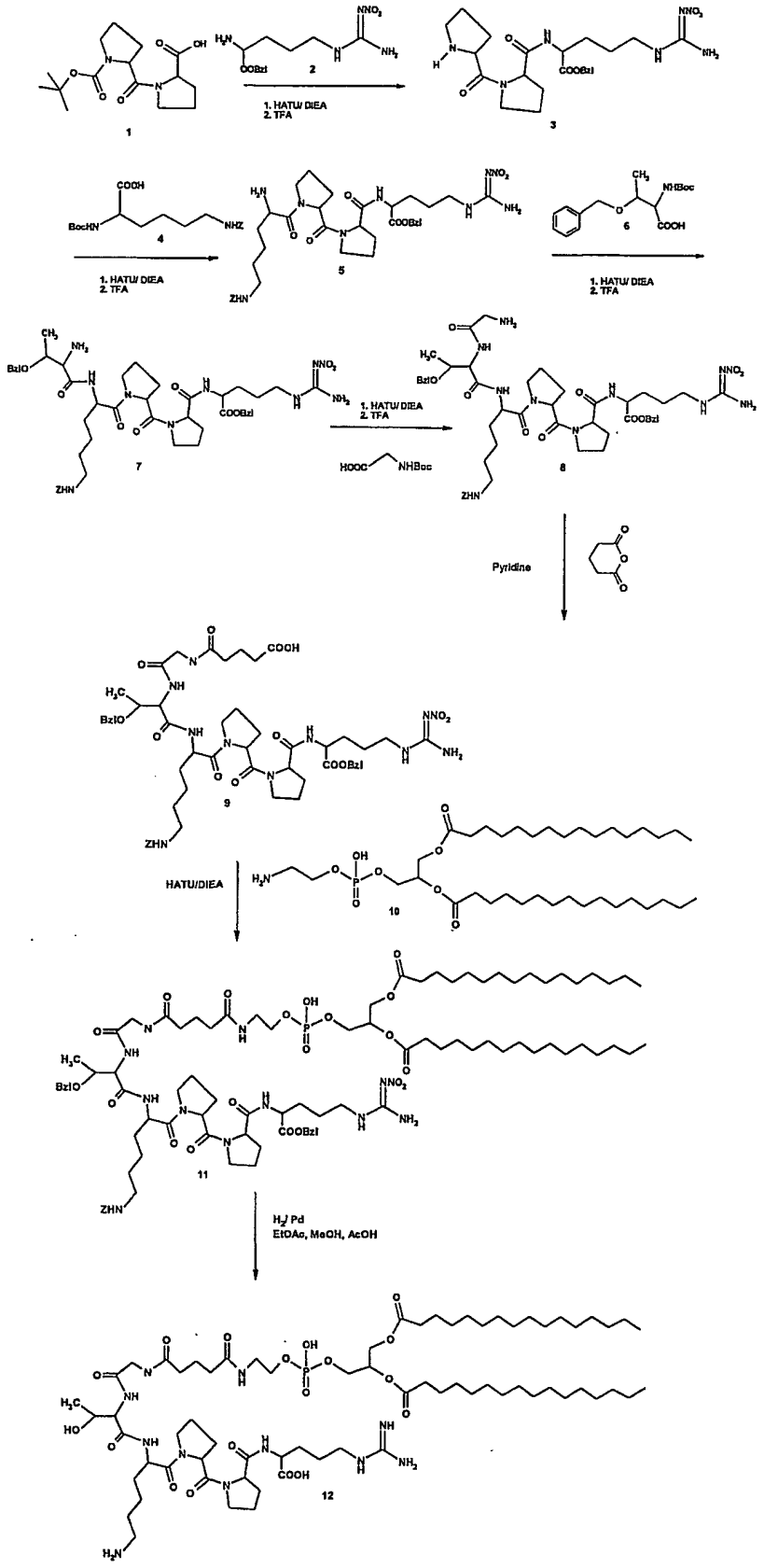
inositol

In a preferred embodiment, A is a multimer of TKPPR such as a TKPPR tetramer.

Particularly preferred are the phospholipids of formula (II) selected from the following group: dimyristoylphosphatidylethanolamine, dipalmitoylphosphatidyl-ethanolamine, distearoylphosphatidylethanolamine or diarachidoylphosphatidyl-ethanolamine; or dioleylphosphatidylethanolamine or dilinoleylphosphatidyl-ethanolamine, fluorinated analogues of any of the foregoing, mixtures of any of the foregoing, with saturated being preferred.

The phospholipids of general formula (II) can be easily coupled, for example, to the compounds of general formula (IIb), as illustrated in the following Scheme 2, for the preparation of a derivative in which in the general formula (IIa), B_1 is dipalmitoylphosphatidylethanolamine and L is constituted by glutaric acid and glycine (see Example 3 of the Experimental section).

Scheme 2



Once the new compounds of general formula (IIa) are synthesized, they can be used as film-forming surfactants for producing the preferred gas-filled microbubble contrast agents of the present invention, together with conventional phospolipids.

The conventional phospholipids correspond to those included in the definition previously given for B_1 and, for example, include any one of lecithins (i.e. phosphatidylcholines), cardiolipin (CL), sphingomyelins, plasmogens, cerebrosides, etc.

The preferred gas-filled microbubbles of the invention can be prepared by means known in the art, such as, for example,by a method described in any one of the following patents: EP 554213, US 5,413,774, US 5,578,292, EP 744962, EP 682530, US 5,556,610, US 5,846,518, US 6,183,725, EP 474833, US 5,271,928, US 5,380,519, US 5,531,980, US 5,567,414, US 5,658,551, US 5,643,553, US 5,911,972, US 6,110,443, US 6,136,293, EP 619743, US 5,445,813, US 5,597,549, US 5,686,060, US 6,187,288, and US 5,908,610.

The disclosure of all of the above-described documents relating to gas-containing contrast agent formulation are incorporated herein by reference.

As disclosed for the first time in EP 474833 (US 5,271,928, US 5,380,519, US 5,531,980, US 5,567,414, US 5,643,553, US 5,658,551, US 5,911,972, US 6,110,443 and US 6,136,293) an aqueous suspension of microbubbles, which may be used in the present invention, is produced with phospholipid film forming surfactants and, optionally, hydrophilic stabilizers. The total concentration of phospholipids in the preferred embodiment of the invention is between 0.01% and 20% and the concentration of microbubbles is preferably between 10^7 and 10^{10} bubbles/mL. The microbubble suspensions of the present invention preferably remain stable for months.

Preferably the concentration of the bubbles of the present invention is between 10^8 and 10^9 bubbles/mL and the concentration of phospholipids used in the microbubbles of the present invention is dependent, in part, on the method of microbubble preparation, the type of phospholipids used for microbubble preparation and the quantity of the peptide or peptide analogue, A, used to achieve stable microbubbles for the ultrasonic contrast agents of the present invention.

The concentration of total phospholipids in a composition of the present invention is preferably in the range of 0.01-10% (w/w) of the total lipid concentration. Most preferred is a range of 0.1-1% (w/w).

In particular the percentage of phospholipids of general formula (IIa) is preferably between 0.1-20 % of total lipids (calculated in mol.%). Most preferred is a range of 0.5-5% (w/w).

Other additives known to those of ordinary skill in the art can be added to the foregoing lipids in admixture with the film forming surfactants in the present invention. For instance, polyoxypropylene glycol and polyoxyethylene glycol and similar compounds, as well as various copolymers thereof; fatty acids such as myristic acid, palmitic acid, stearic acid, arachidic acid or their derivatives, ergosterol, phytosterol, sitosterol, lanosterol, tocopherol, propyl gallate, ascorbyl palmitate and butylated hydroxytoluene may be added. The amount of these non-film forming surfactants is usually up to 50% by weight of the total amount of surfactants but preferably between 0 and 30%.

The microbubble suspensions of the present invention may be prepared from the phospholipids of general formula (IIa) in combination with the conventional phospholipids above defined using already known processes such as a freeze-drying or spray-drying solutions of the crude phospholipids in a suitable solvent. Prior to formation of the suspension by dispersion in an aqueous carrier, the freezedried or spray dried phospholipid powders are contacted with air or another gas. When contacted with the aqueous carrier the powdered phospholipids whose structure has been disrupted will form lamellarized or laminarized segments that will stabilise the microbubbles of the gas dispersed therein. Conveniently, the suspensions of the present invention may also be prepared with phospholipids that were lamellarized or laminarized prior to their contacting with air or another gas. Hence, contacting the phospholipids with air or another gas may be carried out when the phospholipids are in a dry powder form or in the form of a dispersion of laminarized phospholipids in the aqueous carrier.

The introduction of air or gas into a dispersion of laminarized phospholipids in an aqueous carrier (such as, for example, a liposome solution) can be effected by the usual means, injection i.e. forcing air or gas through tiny orifices into the liposome solution, or simply dissolving the gas in the solution by applying pressure and then suddenly releasing the pressure. Another way to introduce air or gas into a dispersion of lamininarized phospholipids in aqueous carrier is to agitate (high shearing homogenisation) or sonicate the liposome solution in the presence of air or another physiologically acceptable gas. Moreover, one can generate the formation of a gas within the solution of liposomes itself, for instance by a gas releasing chemical reaction, e.g. decomposing a dissolved carbonate or bicarbonate by acid.

When laminarized surfactants are suspended in an aqueous liquid carrier and air or another gas is introduced to provide microbubbles, it is thought that the microbubbles become spontaneously or progressively surrounded and stabilised by a monomolecular layer of surfactant molecules and not a bilayer, as in the case of liposome vesicles. This structural rearrangement of the surfactant molecules can be

activated mechanically (agitation) or thermally. The required energy is lower in the presence of non-phospholipid surfactant agents, such as polyoxyethylenepolyoxypropylene block copolymers (e.g. Pluronic® or Synperonic®).

Most preferably, in another approach, non-lamellarized or non-laminarized phospholipids may be obtained by dissolving the phospholipid in an organic solvent and drying the solution without going through the liposome formation stage. In other words, this can be done by dissolving the phospholipids in a suitable organic solvent together with a hydrophilic stabiliser substance e.g. a polymer, like polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), polyethylene glycol (PEG), etc., or a compound soluble both in the organic solvent and water and freeze-drying or spray-drying the solution. Further examples of the hydrophilic stabiliser compounds soluble in water and the organic solvent are malic acid, glycolic acid, maltol and the like. Any suitable organic solvent may be used as long as its boiling point is sufficiently low and its melting point is sufficiently high to facilitate subsequent drying. Typical organic solvents include, for example, dioxane, cyclohexanol, tertiary butanol, tetrachlorodifluoro ethylene ($C_2Cl_4F_2$) or 2-methyl-2-butanol however, 2-methyl-2-butanol and $C_2Cl_4F_2$ are preferred. In this embodiment the criteria used for selection of the hydrophilic stabiliser is its solubility in the organic solvent of choice. The suspensions of microbubbles are produced from such powders using the same steps as with powders of the laminarized phospholipids. Such hydrophilic compounds also aid in homogenising the microbubbles size distribution and enhance stability under storage. Actually making very dilute aqueous solutions (0.0001 - 0.01% by weight) of freeze-dried phospholipids stabilised with, for instance, a 10:1 to 1000:1 weight ratio of polyethyleneglycol to lipid enables the production of aqueous microbubbles suspensions which are stable, without significant observable change, even when stored for prolonged periods. These are obtained by simple dissolution of the air-stored dried laminarized phospholipids without shaking or any violent agitation.

The freeze-drying technique under reduced pressure is very useful because it permits, restoration of the pressure above the dried powders with any physiologically acceptable gas, whereby after redispersion of the phospholipids processed under such conditions suspensions of microbubbles containing the above gases are obtained.

Other gas containing suspensions useful in the invention include those disclosed in, for example, US 5,798,091 (Trevino et al) and WO 97/29783 (designating the US, also EP 881 915), incorporated herein by reference in their entirety. For example, US 5,798,091 discloses what is stated to be a gas emulsion comprising a plurality of bubbles surrounded by a layer of at least a first and a second surfactant. The first surfactant is a hydrophobic phospholipid or mixture of

phospholipids having at least one acyl chain, which comprises at least 10 carbon atoms, and which is at least about 5% w/w of the total surfactant. The second surfactant may or may not also be a phospholipid or mixture of phospholipids, but is more hydrophilic than the phospholipid or combination of phospholipid provided as the first surfactant. Preferred second surfactants may be selected from the group consisting of phospholipids, phosphocholines, lysophospholipids, nonionic surfactants, neutral or anionic surfactants, fluorinated surfactants, which can be neutral or anionic, and combinations of such emulsifying or foaming agents. Some specific examples of surfactants which are useful as the second surfactant include block copolymers of polyoxypropylene and polyoxyethylene (an example of such class of compounds is Pluronic, such as Pluronic F-68), sugar esters, fatty alcohols, aliphatic amine oxides, hyaluronic acid aliphatic esters, hyaluronic acid aliphatic ester salts, dodecyl poly(ethyleneoxy)ethanol, nonylphenoxy poly(ethyleneoxy) ethanol, derivatized starches, hydroxy ethyl starch fatty acid esters, salts of fatty acids, commercial food vegetable starches, dextran fatty acid esters, sorbitol fatty acid esters, gelatin, serum albumins, and combinations thereof. Also contemplated as a second surfactant are polyoxyethylene fatty acids esters, such as polyoxyethylene stearates, polyoxyethylene fatty alcohol ethers, polyoxyethylated sorbitan fatty acid esters, glycerol polyethylene glycol oxystearate, glycerol polyethylene glycol ricinoleate, ethoxylated soybean sterols, ethoxylated castor oils, and the hydrogenated derivatives thereof. In addition, nonionic alkylglucosides such as Tweens ®, Spans ® and Brijs ® may also be used as the second surfactant.

WO 9729783 states that it discloses a contrast agent for use in diagnostic studies comprising a suspension in an injectable aqueous carrier liquid of gas microbubbles stabilised by phospholipid-containing amphiphilic material characterised in that said amphiphilic material consists essentially of phospholipid predominantly comprising molecules with net charges.

WO 9729783 teaches that desirably at least 75%, and preferably substantially all of the phospholipid material in the contrast agents consists of molecules bearing a net overall charge under conditions of preparation and/or use, which charge may be positive or, more preferably, negative. Representative positively charged phospholipids include esters of phosphatidic acids such as dipalmitoylphosphatidic acid or distearoylphosphatidic acid with aminoalcohols such as hydroxyethylenediamine. Examples of negatively charged phospholipids include naturally occurring (e.g. soya bean or egg yolk derived), semisynthetic (e.g. partially or fully hydrogenated) and synthetic phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and cardiolipins. The fatty acyl groups of such phospholipids will typically each contain about 14-22 carbon atoms, for example

as in palmitoyl and stearoyl groups. Lyso forms of such charged phospholipids are also useful, the term "lyso" denoting phospholipids containing only one fatty acyl group, this preferably being ester-linked to the 1position carbon atom of the glyceryl moiety. Such lyso forms of charged phospholipids may advantageously be used in admixture with charged phospholipids containing two fatty acyl groups.

These agents may be prepared as described in US 5,798,091 or WO97/29783. For example, US 5,798,091 teaches that contrast agents may be prepared by first dispersing, in an aqueous solution, a hydrophilic monomer or polymer or combination thereof, a first and a second surfactant, and an inflating agent. As discussed supra, the first surfactant is stated to be a phospholipid or mixture of phospholipids having at least one acyl chain comprising at least 10 carbon atoms and comprising at least about 5% w/w of total surfactant, and the second surfactant is more water-soluble than said first surfactant.The dispersion is then spray dried to evaporate the inflating agent and to create what is described as a dry, hollow, particulate, approximately microspherical material. This dry particulate material is exposed to at least a first gas, and then may be dissolved in an aqueous liquid, thereby forming what is described as an aqueous gas emulsion composition. The patent states that the composition comprises bubbles of the gas surrounded by a layer of the first and second surfactants, and that the stability is independent of liposomes.

These contrast agents may also be prepared according to WO 9729783. WO 9729783 states that these agents may be prepared by a process, comprising the steps of:

i) generating a dispersion of gas microbubbles in an aqueous medium containing what is described as a membrane-forming lipid;

ii) lyophilising the thus-obtained lipid stabilised gas dispersion to yield a dried lipid containing product; and

iii) reconstituting the dried product in an injectable aqueous carrier liquid.

It is stated that step (i) may, for example, be effected by subjecting the lipid-containing aqueous medium to any appropriate emulsion-generating technique, for example sonication, shaking, high pressure homogenisation, high speed stirring or high shear mixing, e.g. using a rotorstator homogeniser, in the presence of the selected gas. The aqueous medium may, if desired, contain additives, which serve as viscosity enhancers and/or as solubility aids for the lipid, such as alcohols or polyols, e.g.glycerol and/or propylene glycol.

Any biocompatible gas may be present in the agents of the present invention, the term "gas" as used herein including any substances (including mixtures) substantially in gaseous form at the normal human body temperature. The gas may

thus include, for example, air; nitrogen; oxygen; CO_2; argon; xenon or krypton, fluorinated gases (including for example, perfluorocarbons, SF_6, SeF_6) a low molecular weight hydrocarbon (e.g. containing from 1 to 7 carbon atoms) for example an alkane such as methane, ethane, a propane, a butane or a pentane, a cycloalkane such as cyclopropane, cyclobutane or cyclopentene, an alkene such as ethylene, propene, propadiene or a butene, or an alkyne such as acetylene or propyne and/or mixtures thereof.

Fluorinated gases are preferred. Fluorinated gases include materials which contain at least one fluorine atom such as SF_6, freons (organic compounds containing one or more carbon atoms and fluorine, i.e. CF_4, C_2F_6, C_3F_8, C_4F_8,C_4F_{10},$CBrF_3$, CCl_2F_2,C_2ClF_5, and $CBrClF_2$) and perfluorocarbons. The term perfluorocarbon refers to compounds containing only carbon and fluorine atoms and includes, in particular, saturated, unsaturated, and cyclic perfluorocarbons. The saturated perfluorocarbons, which are usually preferred, have the formula C_nF_{n+2}, where n is from 1 to 12, preferably from 2 to 10, most preferably from 3 to 8 and even more preferably from 3 to 6. Suitable perfluorocarbons include, for example, CF_4, C_2F_6, C_3F_8 C_4F_8, C_4F_{10}, C_5F_{12}, C_6F_{12}, C_7F_{14}, C_8F_{18}, and C_9F_{20}. Preferably the gas or gas mixture comprises SF_6 or a perfluorocarbon selected from the group consisting of C_3F_8 C_4F_8, C_4F_{10}, C_5F_{12}, C_6F_{12}, C_7F_{14}, C_8F_{18}, with C_4F_{10} being particularly preferred.

As cited above the gas can be a mixture of the gases, as defined above. In particular the following combinations are particularly preferred: a mixture of gases (A) and (B) in which, at least one of the gases (B), present in an amount of between 0.5 - 41% by vol., has a molecular weight greater than 80 daltons and (B) is selected from the group consisting of SF_6, CF_4, C_2F_6, C_2F_8, C_3F_6, C_3F_8, C_4F_6, C_4F_8, C_4F_{10}, C_5F_{10}, C_5F_{12} and mixtures thereof and (A) is selected from the group consisting of air, oxygen, nitrogen, carbon dioxide and mixtures thereof the balance of the mixture being gas A.

In certain circumstances it may be desirable to include a precursor to a gaseous substance (e.g. a material that is capable of being converted to a gas in vivo). Preferably the gaseous precursor and the gas it produces are physiologically acceptable. The gaseous precursor may be pH-activated, photo-activated, temperature activated, etc. For example, certain perfluorocarbons may be used as temperature activated gaseous precursors. These perfluorocarbons, such as perfluoropentane, have a liquid/gas phase transition temperature above room temperature (or the temperature at which the agents are produced and/or stored) but below body temperature; thus they undergo a phase shift and re converted to a gas within the human body.

In practice, all injectable compositions should also be ,as far as possible ,isotonic with blood. Hence, before injection, small amounts of isotonic agents may also be added to the suspensions of the invention. The isotonic agents are physiological solutions commonly used in medicine and they comprise aqueous saline solution (0.9% NaCl), 2.6% glycerol solution, 5% dextrose solution, etc.

A preferred embodiment of the method of the present invention includes selecting a film forming surfactant and optionally converting it into lamellar form using one of the methods known in the art or disclosed hereinbefore. The surfactant is then contacted with air or another gas and admixed with an aqueous liquid carrier in a closed container whereby a suspension of microbubbles will form. The suspension is allowed to stand for a while and a layer of gas filled microbubbles formed is left to rise to the top of the container. The lower part of the mother liquor is then removed and the supernatant layer of microbubbles washed with an aqueous solution saturated with the gas used in preparation of the microbubbles. This washing can be repeated several times until substantially all unused or free surfactant molecules are removed. Unused or free molecules means all surfactant molecules that do not participate in formation of the stabilising monomolecular layer around the gas microbubbles.

The gas-containing microbubbles formulations containing the targeting moiety of the present invention may be prepared by reconstitution from the dry powder by a suitable physiologically acceptable aqueous carrier, such as buffered or unbuffered physiological saline solution (0.9% aqueous NaCl; buffer 10 mM tris-HCl) or a 5% aqueous dextrose or mannitol solution or a 2.6% aqueous glycerol solution. When the manufacture of injectable therapeutically effective compositions comprising the microbubbles of the invention are contemplated, the microbubbles carrying active ingredients are suspended in the commonly used physiologically acceptable carriers containing known additives and stabilizers.

The microbubbles of the invention may also be used for the delivery of therapeutically active substances, in which case the active substance may be included in the membrane. The compounds of general formula (Ia) or (IIa) are particularly suitable for incorporation into lipidic or lipidic/polymeric membrane material. The amount of lipophilic active material incorporated into the membrane will depend on the nature and the molecular weight; however, very high active substance to lipid ratios are obtained when lipophilic substances are used. Virtually any biologically active substance useful for the therapeutic applications of the present invention can be used with the microbubbles according to the invention. Such substances include but are not limited to, antineoplastic, antiangiogenic, angiogenic compounds, anti-inflammatory compounds, genes, antisense compounds etc.

In another aspect, the present invention relates to agents based on microcapsules/microballoons (microballoons) in which the new compounds of general formula (I) and more particularly (IIa) may be incorporated. As discussed, the term "microballoon" refers to gas filled bodies with a material boundary or envelope. Gas-filled liposomes according to, for example, US 5,123,414 (Unger) also belong to this category and are incorporated herein by reference. More on these different formulation my be found in EP-A-0 324 938 (US 4,844,882, Widder et al.), US 5,711,933 (Bichon et al.), US 4,900,540 (Ryan), US 5,230,882 (Unger), 5,469,854 (Unger), 5,585,112 (Unger), Us 4,718,433 (Feinstein), US 4774,958 (Feinstein), WO 9501187 (MBI designating the US), US 5,529,766 (Nycomed), US 5,536,490 (Nycomed), US 5,990,263 (Nycomed), the content of which are incorporated herein by reference.

The preferred microballoons of the present invention have the envelope constituted by B_3, a biodegradable physiologically compatible polymer or B_{3a}, a biodegradable solid lipid.

The polymers B_3 useful for the preparation of the microballoons of the present invention can be selected from the biodegradable physiologically compatible polymers, such as any of those described in any of the following patents: EP 458745, US 5,711,933, US 5,840,275, EP 554213, US 5,413,774 and US 5,578,292, the entire contents of each of which are incorporated herein by reference. In particular, the polymer which constitutes the envelope or bounding membrane can be selected from biodegradable physiologically compatible polymers, such as polysaccharides of low water solubility, polylactides and polyglycolides and their copolymers, copolymers of lactides and lactones such as e-caprolactone, γ-valerolactone and polypeptides. The great versatility in the selection of synthetic polymers is another advantage of the present invention since, as with sensitive patients, one may wish to avoid using microballoons made of natural proteins (albumin, gelatin) as in US 4,276,885 or EP-A-324.938. Other suitable polymers include poly(ortho)esters (see for instance US 4,093,709; US 4,131,648; US 4,138,344; US 4,180,646); polylactic and polyglycolic acid and their copolymers, for instance DEXON (see J. Heller, Biomaterials 1 (1980), 51; poly(DL-lactide-co- e-caprolactone), poly(DL-lactide-co- γ-valerolactone), poly(DL-lactide-co- γ-butyrolactone), polyalkylcyanoacrylates; polyamides, polyhydroxybutyrate; poly-dioxanone; poly-ß-aminoketones (Polymer 23 (1982), 1693); polyphosphazenes (Science 193 (1976), 1214); and polyanhydrides. References on biodegradable polymers can be found in R. Langer et al., Macromol. Chem. Phys. C23 (1983), 61-126. Polyamino-acids such as polyglutamic and polyaspartic acids can also be used as well as their derivatives, i.e. partial esters with lower alcohols or glycols. One

useful example of such polymers is poly(t.butyl-glutamate). Copolymers with other aminoacids such as methionine, leucine, valine, proline, glycine, alamine, etc. are also possible. Recently, novel derivatives of polyglutamic and polyaspartic acid with controlled biodegradability have been reported (see WO 87/03891; US 4,888,398 and EP-130.935, incorporated here by reference). The lipids B_{3a} useful in the present including are discussed infra.

The microballoons which may be particularly useful in certain applications of the present invention are pressure sustaining microballoons bounded by a soft and elastic membrane which can temporarily deform under variations of pressure and are endowed with enhanced echogenicity and are biodegradable.

The amount of the compounds of general formula (I) that may be incorporated in the microballoon of the present invention may vary depending, for example, on the particular polymer B_3 or lipid B_{3a} involved. In certain preferred embodiments, the microballoons composition comprising the targeting moiety may comprise as low as 0.5% mol. of a compound of general formula (I) up to 50% of the total polymer B_3 or the lipid B_{3a}. The more preferred range is between 5% and 15% of the total.

The microballoons of the present invention are preferably prepared by emulsifying with an emulsifier a hydrophobic phase in an aqueous phase (usually containing viscosity increasing agents as emulsion stabilizers) thus obtaining an oil-in-water type emulsion of droplets of the hydrophobic phase and thereafter adding thereto a membrane forming polymer dissolved in a volatile organic solvent not miscible with the aqueous phase.

Known techniques can be adapted to the preparation of air or gas filled microballoons suited for ultrasonic imaging, according to the present invention, provided that appropriate conditions are found to control sphere size in the desired ranges, balloon-wall permeability or imperviousness and replacement of the encapsulated liquid phase by air or a selected gas. Control of overall sphere size is important to adapt the microballoons for their intended use, i.e. parenteral administration (about 0.5 - 10 μm average size). Control of balloon-wall permeability is important to ensure that injectable aqueous carrier phase does not infiltrate or infiltrates at a slow enough rate so as not to impair the echographic measurements but is still sufficient to ensure relatively fast after-test biodegradability, i.e. ready metabolization of the suspension by the organism. Also the microporous structure of the microballoons envelope (pores of a few nm to a few hundreds of nm or more for microballoons envelopes of thickness ranging from 50-500 nm) influences their resiliency, i.e. the microspheres can readily accept pressure variations without breaking. The preferred range of pore sizes is about 50-2000 nm.

A preferred method for forming the microballoons with a biodegradable envelope constituted by polymers B_3 in mixture with the compounds (Ia) of the present invention, is as follows:

emulsifying a hydrophobic organic phase into a water phase so as to obtain droplets of said hydrophobic phase as an oil-in-water emulsion in the water phase;

adding to the emulsion a solution of a polymer together with the compounds of general formula (I) in a volatile solvent insoluble in the water phase, so that a layer of the polymer will form around the droplets;

evaporating the volatile solvent so that the polymer will deposit by interfacial precipitation around the droplets which then form beads with a core of the hydrophobic phase encapsulated by a membrane of the polymer, the beads being in suspension in the water phase; and

subjecting the suspension to reduced pressure under conditions such that the encapsulated hydrophobic phase is removed by evaporation.

Preferably, the hydrophobic phase is selected so that the hydrophobic phase evaporates under reduced pressure substantially simultaneously with the water phase and is replaced by air or gas, whereby dry, free flowing, readily dispersible microballoons are obtained. More preferably, the addition of the polymer and evaporation of the volatile solvent steps can be omitted and the polymer membrane will be formed by interfacial precipitation during the application of a reduced pressure.

One factor which enables control of the permeability of the microballoon membrane is the rate of evaporation of the hydrophobic phase relative to that of water during the application of reduced pressure in the above method, e.g. under conditions of freeze drying which is the case of the embodiment recited below. For instance, if the evaporation is carried out between about –40°C and 0°C, and hexane is used as the hydrophobic phase, 50:50 DL-lactide/glycolide copolymer being the interfacially deposited polymer, beads with relatively large pores are obtained due to the vapour pressure of the hydrocarbon in the chosen temperature range which is significantly greater than that of water. This creates a condition whereby the pressure difference between the inside and outside of the spheres will tend to increase the size of the pores in the membrane through which the inside material will be evaporated. In contrast, using cyclooctane as the hydrophobic phase (which has a vapor pressure of -17°C, which is the same as that of water) will provide beads with very tiny pores because the difference of pressures between the inside and outside of the spheres during evaporation is minimised.

Depending on the degree of porosity desired, the microballoons of this invention can be made stable in an aqueous carrier from several hours to several months and give reproducible echographic signals for a long period of time. Actually, depending on the polymer selected, the membrane of the microballoons can be made substantially impervious when suspended in carrier liquids of appropriate osmotic properties, i.e. containing solutes in appropriate concentrations. It should be noted that the existence of micropores in the envelope of the microballoons of the present invention appears to be also related with the echographic response, i.e., all other factors being constant, microporous vesicles provide more efficient echographic signal than corresponding non-porous vesicles. Other water-insoluble soluble organic solvents which have a vapour pressure of the same order of magnitude between about -40°C and 0°C are convenient as hydrophobic solvents in this invention. These include hydrocarbons such as, for instance, n-octane, cyclooctane, the dimethylcyclohexanes, ethyl-cyclohexane, 2-, 3- and 4-methyl-heptane, 3-ethyl-hexane, toluene, xylene, 2-methyl-2-heptane, 2,2,3,3-tetramethylbutane and the like. Esters, such as propyl and isopropyl butyrate and isobutyrate, butyl-formate and the like, are also convenient in this range. Another advantage of freeze drying is to operate under reduced pressure of a gas instead of air, whereby gas filled microballoons will result. Physiologically acceptable gases are those cited above for the gas-filled microbubbles. Gases with radioactive tracer activity can be contemplated.

As the volatile, water-insoluble solvent to be used for dissolving the polymer to be precipitated interfacially, one may also use halo-compounds such as CCl_4, CH_3Br, CH_2Cl_2, chloroform, perfluorocarbons as defined above, low boiling esters such as methyl, ethyl and propyl acetate as well as lower ethers and ketones of low water solubility. When solvents which are not totally insoluble in water are used, e.g. diethyl-ether, it is advantageous to use, as the aqueous phase, a water solution saturated with said solvent beforehand.

The aqueous phase in which the hydrophobic phase is emulsified as an oil-in-water emulsion preferably contains 1-20% by weight of water-soluble hydrophilic compound(s), such as sugars and polymers as stabilizers, e.g. polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), gelatin, polyglutamic acid, albumin, and polysaccharides such as starch, dextran, agar, xanthan and the like. Similar aqueous phases can be used as the carrier liquid in which the microballoons are suspended before use.

Part of this water-soluble polymer can remain in the envelope of the microballoons or it can be removed by washing them before subjecting to final evaporation of the encapsulated hydrophobic core phase.

The emulsifiers to be used (0.1-5% by weight) to provide the oil-in-water emulsion of the hydrophobic phase in the aqueous phase include most physiologically acceptable emulsifiers, for instance the phosholipids defined above. Emulsifiers also include surfactants such as free fatty acids, esters of fatty acids with polyoxyalkylene compounds like polyoxypropylene glycol and polyoxyethylene glycol; ethers of fatty alcohols with polyoxyalkylene glycols; esters of fatty acids with polyoxyalkylated sorbitan; soaps; glycerol-polyalkylene stearate; glycerol-polyoxyethylene ricinoleate; homo- and copolymers of polyalkylene glycols; polyethoxylated soya-oil and castor oil as well as hydrogenated derivatives; ethers and esters of sucrose or other carbohydrates with fatty acids, fatty alcohols, these being optionally polyoxyalkylated; mono-, di- and triglycerides of saturated or unsaturated fatty acids; glycerides or soya-oil and sucrose.

Additives can be incorporated into the polymer membrane of the microballoons to modify the physical properties such as dispersibility, elasticity and water permeability. For incorporation in the polymer, the additives can be dissolved in the polymer carrying phase, e.g. the hydrophobic phase to be emulsified in the water phase, whereby they will co-precipitate with the polymer during inter-facial membrane formation.

Useful additives may include compounds which can "hydrophobize" the microballoon membrane in order to decrease water permeability, such as fats, waxes and high molecular-weight hydrocarbons. Additives which improve dispersibility of the microballoons in the injectable liquid-carrier, and may be included in the compositions of the present invention, include amphipathic compounds like the phospholipids.The amphipathic compounds may also increase water permeability and/or the rate of biodegradability.

Additives which increase membrane elasticity, and may be included in the compositions of the present invention, include plasticizers, like isopropyl myristate and the like. Also, very useful additives are constituted by polymers akin to that of the membrane itself but with relatively low molecular weight. For instance when using copolymers of polylactic/polyglycolic type as the membrane forming material, the properties of the membrane can be modified advantageously (enhanced softness and biodegradability) by incorporating, as additives, low molecular weight (1000 to 15,000 Dalton) polyglycolides or polylactides. Also polyethylene glycol of moderate to low M_w (e.g. PEG 2000) is a useful softening additive.

Sterols are preferably used in admixture with the other glycerides and or fatty acids and are selected from cholesterol, phytosterol, lanosterol, ergosterol, etc. and esters of the sterols with the above mentioned fatty acids; however, cholesterol is preferred.

The microballoons of the present invention can also be prepared according to the methods of WO-A-96/15815, and, i.e. on the unexpected finding that a particularly useful solid microcapsule with a mean size from a fraction of micrometer to 1000 micrometers may be obtained when one or more biodegradable solid lipids, at room temperature, are used to encapsulate a core which comprises air or a gas. Useful biodegradable lipids B_{3a} are solid water insoluble mono-, di- or tri-glycerides, fatty acids, fatty acid esters, sterols such as cholesterol, waxes and mixtures thereof. Mono-, di- and tri- glycerides include mainly the mono-, di- and tri-laurin compounds as well as the corresponding -myristin, -palmitin, -stearin, -arachidin and -behenin derivatives. Mono-, di- and tri- myristin, -palmitin -stearin and mixed triglycerides such as dipalmitoylmonooleyl glyceride are particularly useful; however, tripalmitin and tristearin are preferred. When made from fatty acids or mixtures of fatty acids with glycerides and/or sterols, the fatty acids include all, at room temperature solid, fatty acids (preferably saturated) having 12 carbon atoms or more. These fatty acids include, for instance, lauric, arachidic, behenic, palmitic, stearic, sebacic, myristic, cerotinic, melissic and erucic acids, the fatty acid esters. Preferably, the fatty acids and their esters are used in admixture with other glycerides.

A preferred microballoon composition was obtained with triglycerides such as tripalmitin, tristearin or mixtures of the above mentioned triglycerides. Lower yields and microballoons with a slight tendency to agglomeration were obtained when diglycerides were used. The lowest yields of microballoons were obtained with monoglycerides. The degree of hydrophobicity appears to explain the fact that the best microballoons are obtained from the fairly hydrophobic materials and as the hydrophobicity decreases or surface activity increases the quality and the quantity of the microballoons obtained decreases. The greater participation of the more hydrophobic triglyceride (lipid) the better the microballoon yield and the smoother the process of the manufacture.

Optionally, biodegradable water insoluble lipids may be admixed with up to 75% by weight of biodegradable polymers. The amount of biodegradable polymers is limited to 75% by weight, because the biodegradability of the glyceride/polymer mixtures is not a linear function of the composition i.e. the biodegradability does not increase or decrease in direct proportion to the amount of the polymer present in the mixture, but that it is more determined or influenced by the biodegradability of the glycerides than by that of the polymers. This is so only as long as the amount of glycerides is equal to or greater than 25% by wt. as the mixtures containing 25% by wt. or more of the glyceride have biodegradability closer to that of lipids than to that of polymers. However, the mixtures with 75% by wt. or more of the polymer have biodegradability closer to that of pure polymers. This means that the mixtures with

less than 25% of glycerides in terms of biodegradability will behave almost like the pure polymers. When, however, the amount of lipids approaches 25% the character of the mixture changes and further increase of the amount of lipids has a greater impact on the biodegradability of the mixture by imposing the lipid biodegradability rate on the polymers, i.e. rendering the mixture more biodegradable than what would or could be expected considering the amount of polymer present. This clearly demonstrates that biodegradability of the mixture is not a simple sum of the individual biodegradabilities but is conditioned by the component present in excess, however in such a way that the influence of the glycerides is predominant. For compositions with more than 75% by weight of the polymer, biodegradability rapidly approaches that of the pure polymer.

The glyceride containing hollow microballoons of the present invention preferably are prepared with an average size between 0.1 μm and 1000 μm by dispersing, in an aqueous carrier phase, a mixture of one or more of the solid constituents of the microcapsule envelope dissolved in an organic solvent, so as to produce an oil-in-water emulsion. The emulsion water phase may contain an effective amount of surfactants which are used to stabilise the emulsion. Surfactants such as (PVA), polyoxyethylene-polyoxypropylene block copolymers, phospholipids such as phosphatidic acid, phosphatidyl choline, phosphatidylethanol amine, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol and mixtures thereof, sorbitan ethers, sorbitan esters, polyoxyethylenesorbitan esters, ethoxylated saturated glycerides and partial fatty acid glycerides or polyglycerides, etc., may be used, but polyoxyethylene-polyoxypropylene block copolymers (e.g. Pluronic®, or Synperonic®) and phospholipids are preferred. The presence of the surfactants is compulsory only if the size of the final product or particle size distribution is important. If the preparation is intended for the parental administration, presence of the surfactant in the water phase is important. Prior to freezing at a temperature below -30°C, a certain amount of redispersing agent is added to the emulsion of tiny droplets of the organic solution in the water phase. The frozen emulsion is then subjected to reduced pressure to effect lyophilisation, i.e. the removal by sublimation of the organic solvent from the droplets and of the water of the carrier phase. Without wishing to be bound by any particular theory, it is postulated that during this relatively slow solvent removal, the membrane constituents migrate outwardly to the periphery of droplets until they arrive to the frozen water boundary where their further motion is impeded causing the formation of a molecularly organized dense deposit at the solvent/ice interface which may acquire a semi-crystalline structure in the area at the junction between the solvent and the ice, i.e. at the solvent to ice interface.

Any convenient redispersing agent may be used; however redispersing agents selected from albumin, gelatine, PVP, PVA, PEG and polyoxyethylene-polyoxypropylene block copolymer are preferred. The redispersing agents which are added to prevent particle agglomeration are particularly useful when the microballoons are in the form of non-coalescent, dry and instantly dispersible powders. Produced for a long storage or from hydrophobic triglyceride materials such as tripalmitin or tristearin, the microballoons preparations of the invention further comprise one or more redispersing agents. Where the microballoons comprise gas filled liposomes, they may be prepared as described in, for example, US 5,123,414, US 5,469,854, US 5,585,112, and WO 9222247 (Unger) (designating the US), incorporated herein by reference in their entirety, and adapted to include the targeting moiety of the invention as discussed herein.

The porosity of the hollow microballoons made according to the invention is usually very low and sometimes the microballoons have no pores at all. It appears that the porosity is a function of the lipid concentration or wall thickness of the microcapsule. When porous, the microballoons of the invention have a pore size in the range of 20 to 2,000 nm.

As already mentioned when the microballoons of the invention are prepared from mixtures of one or more biodegradable water insoluble lipids B_{3a} with biodegradable polymers B_3, up to 75% by weight of the polymer may be used. Microballoons of controlled half-life after administration can be customized by adjusting the respective proportions of the lipids B_{3a} and biodegradable polymers B_3 during fabrication. The exact amount of the polymer will depend on the application and will be directly related to the degree of biodegradability required. For example, for certain sustained release applications the amount of biodegradable polymer may be anywhere between 30% and 60% by wt. and in some cases up to 75% by weight. However, if the microballoons of the invention are used for echographic imaging, depending on the desired rate of clearance from the body, the amount of biodegradable polymer may be between 1-50% by wt. preferably between 0.5-10% by wt. or as low as 0.1% by wt.

The microballoons used for echography typically having relatively thin walls (e.g. 50-500 nm thick)are particularly advantageous as their biodegradability is very rapid (i.e. the clearance of the lipidic envelopes from the body, occurs within a relatively short period of time).

When microballoons are made from mixtures of one or more water insoluble lipids B_{3a} with a biodegradable polymer B_3 as defined previously, however, polylactides and polyglycolides and their copolymers are preferred.

The microballoons of the invention may be used for the delivery of therapeutically active substances, in which case the active substance may be included in the membrane or may be loaded in the core. The compounds of general formula (Ia) or (IIa) are particularly suitable for incorporation into lipidic or lipidic/polymeric membrane material. The amount of lipophilic active material incorporated into the membrane will depend on the nature and the molecular weight; however, very high active substance to lipid ratios are obtained when lipophilic substances are used. Virtually any biologically active substance useful for the therapeutic applications of the present invention can be used with the microballoons according to the invention. Such substances include but are not limited to, antineoplastic, antiangiogenic, angiogenic compounds, anti-inflammatory compounds, genes, antisense compounds etc.

Experiments have shown that when the microballoons of the invention are used as delivery vehicles for active substances, different effects may be achieved by varying the concentration of the lipid or lipid/polymer mixture in the starting material. It has been established that microballoons with relatively thin walls and a high active substance to lipid or lipid/polymer ratio, i.e. high concentration of the active ingredient, will produce a shock treatment in the surrounding tissue. A particular advantage of the microballoons of the invention comes from the fact that the shock treatment may be customized by varying the ratio or the wall thickness while maintaining the concentration of the active substance at a constant level thus producing a form of sustained release system. The system in turn may be fully adapted to the substance carried, the treatment envisaged and even the physiological condition of the patient.

The present invention provides injectable compositions including a suspension of an effective amount of microballoons in a pharmaceutically acceptable liquid carrier with optional additives known to those of ordinary skill in the art and stabilisers.

Echographic contrast agents are readily produced by suspending the microballoons of the invention in a suitable physiologically acceptable aqueous carrier, such as buffered or unbuffered physiological saline solution (0.9% aqueous NaCl; buffer 10 mM tris-HCl) or a 5% aqueous dextrose or mannitol solution or a 2.6% aqueous glycerol solution. When the manufacture of injectable therapeutically effective compositions comprising the microballoons of the invention are contemplated, the microballoons carrying active ingredients are suspended in the commonly used physiologically acceptable carriers containing known additives and stabilizers.

Other useful gas-containing contrast agent formulations include gas-containing solid systems, for example microcparticles (especially aggregates of microparticles) having gas contained therein or othewise associated therewith (for particles (especially aggregates of microparticles) having gas contained therein or othewise associated therewith (for example being adsorbed on the surface thereof and/or contained within voids, cavities or pores therein). These contrast agents may be adapted to contain the targeting moiety of the invention as discribed herein. Methods for the preparation of these agents are as described in EP 0122624 EP 0123235, EP 0365467, US 5,558,857, US 5,607,661, US 5,637,289, US 5,558,856, US 5,137,928, WO 9521631 or WO 9313809, incorporated herein by reference in their entirety. It will be appreciated that the echogenicity of these contrast agents may derive directly from the contained/associated gas and/or from gas (e.g. escribed herein. Methods for the preparation of these agents are as described in EP 0122624 EP 0123235, EP 0365467, US 5,558,857, US 5,607,661, US 5,637,289, US 5,558,856, US 5,137,928, WO 9521631 or WO 9313809, incorporated herein by reference in their entirety. It will be appreciated that the echogenicity of these contrast agents may derive directly from the contained/associated gas and/or from gas (e.g. microbubbles) liberated from the solid material (e.g. upon dissolution of the microparticulate structure).

In another aspect of the present invention, we have found a new model in vitro and in vivo (only for animals) for the screening of the agents of the present invention: the compounds are attached to polymer beads or other non-lipid polymer materials which are labeled with a detectable label (e.g. a flourescent dye).

Typically, in vitro screening of compounds for binding to a desired site is accomplished by incubating a labeled (radioactive, fluorescent, etc.) form of the compound with an appropriate in vitro model. Such assays developed to measure compound binding have some limitations. First, the sensitivity of the assay is often so low that binding cannot be easily detected or quantitated. Because of this, many screening assays utilize whole cells or membrane fractions from cell lines engineered to overexpress the binding target. If the exact binding target is unknown, or is not easily expressed through genetic methods, this approach is not feasible. Secondly, the assay will usually only detect relatively high-affinity binding events. This means that the opportunity to identify moderately tight-binding compounds, which can then be optimized to improve binding, is lost.

However attaching compounds to beads or other particles can largely overcome the limitations of screening with labeled compounds. A single fluorescently labeled bead, with a diameterattaching compounds to beads or other particles can largely overcome the limitations of screening with labeled compounds. A single fluorescently

labeled bead, with a diameter of about 1 micron or greater, can easily be seen using an ordinary fluorescent microscope. For higher throughput screening, fluorescent beads can easily be detected and quantitated on fluorescence microplate readers. Alternatively, easily detectable amounts of radioactivity can be incorporated into the agents of the invention. Assays using such radioactive entities would be more sensitive than those utilizing individually labeled compound molecules.

Another advantage of attaching compounds to beads for screening purposes is that numerous molecules of the compound become attached per bead. The resulting multivalent presentation of the molecule increases the binding avidity of the bead for its target, and allows the detection of compounds that might not be identified by more traditional screening assays, due to a relatively low binding strength of the compound as a single molecule.

Furthermore, access to the targeting moiety on the agents of the invention may be modified because they are presented on the surface of a large entity. Thus the targeting moiety may interact with different targets, when bound to microvesicles or beads, then when as small molecules or individual molecules. Thus another aspect of the invention is the use of easily prepared beads to predict the behavior of similarly derivatized microspheres.of about 1 micron or greater, can easily be seen using an ordinary fluorescent microscope. For higher throughput screening, fluorescent beads can easily be detected and quantitated on fluorescence microplate readers. Alternatively, easily detectable amounts of radioactivity can be incorporated into the agents of the invention. Assays using such radioactive entities would be more sensitive than those utilizing individually labeled compound molecules.

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Furthermore, access to the targeting moiety on the agents of the invention may be modified because they are presented on the surface of a large entity. Thus the targeting moiety may interact with different targets, when bound to microvesicles or beads, than when as small molecules or individual molecules. Thus another aspect of the invention is the use of easily prepared beads to predict the behavior of similarly derivatized microspheres.

Thus, the instant invention includes attaching monomers, multimers or polymers of TKPPR, (or a TKPPR analogue) to beads for use in screening and other

assays. In this embodiment, the present invention provides compounds of general formula (Ib)

$$A\text{-}L\text{-}B_2 \qquad (Ib)$$

in which

B_2 is a non-lipid polymer able to bind the linker in a covalent manner, and A and L have the same meanings above defined.

In preferred embodiment of the present invention, B_2 corresponds to B_{2a}, a polymer which can be used for producing microparticles or beads containing functional groups, such as acid or amino groups, able to bind chemical entities or B_2 is the bead itself. Microparticles are generally considered to be spherical or irregular in shape, and to be less than about 50 micrometers in diameter. They may be prepared by several practical methods from a variety of polymerizable monomers, including styrenes, acrylates and unsaturated chlorides, esters, acetates, amides and alcohols. Microparticles can be further modified by coating with one or more secondary polymers to alter the surface properties of the particles.

In a more preferred embodiment of the present invention the bead is a commercially available bead which is derivatizable and may, optionally include a detectable label. The detectable label may preferably be one which generates light or a radioactive signal, such as are known in the art.

Beads labeled with fluorescent dyes, for example, have found use in a wide variety of applications. Fluorescent beads are most commonly used in applications that can benefit from use of monodisperse, chemically inert, biocompatible particles that emit detectable fluorescence and that can bind to a particular substance in the environment. For example, fluorescent particles to which biological molecules have been attached have been used for immunoassays (U.S. Pat. No. 4,808,524 (1989)), for nucleic acid detection and sequencing (Vener, et al. ANALYT. BIOCHEM. 198, 308 (1991); Kremsky, et al., NUCLEIC ACIDS RES. 15, 2891 (1987); Wolf, et al., NUCLEIC ACIDS RES. 15, 2911 (1987)), as labels for cell surface antigens, FLOW CYTOMETRY AND SORTING, ch. 20 (2@nd ed. (1990)), and as tracer to study cellular metabolic processes (J. LEUCOCYTE BIOL. 45, 277 (1989)). The high surface area of beads provides an excellent matrix for attaching chemical entities, such as the compounds of general formula (IIb), or A, by using a classical condensing agent, while the fluorescent properties of these particles enable them to be detected with high sensitivity. They can be quantitated by their fluorescence either in aqueous suspension or when captured on membranes.

Fluorescent beads can be visualized with a variety of imaging techniques, including ordinary light or fluorescence microscopy and laser scanning confocal

microscopy as well as fluorescent/flow activated cell sorters (FACS) which may optionally containing cell sorting capabilities.

The most preferred beads of the present invention are fluorescent, 2μ diameter beads having carboxy or amino groups that can be derivatized as known by those skilled in the art, such as those from Molecular Probes Inc.

In the present invention peptide A or its analogues provide sufficient binding strength to attach ultrasound agents, such as microbubbles or microballons or beads to cultured HAEC (Human aortic endothelial cells) under flow. Where desirable, the binding strength and stability of lead compounds can be optimized using methods known to those of ordinary skill in the art. In a preferred embodiment of the invention, to facilitate the attachment of compound libraries to beads, the compounds can be synthesized with a tag, such as biotin, which will tightly associate with an acceptor molecule on the beads, such as avidin or vice versa.

The beads do not necessarily have to be labeled with fluorescence or radioactivity. They can be prepared such that they can be detected and quantified by visible light methods (colorimetry), or contain an enzyme or other signal generating system known to those skilled in the art that can be activated after specific binding to a target has occurred.

Beads can be reacted with the compounds of general formula (IIb) to give the agents of general formula (Ib). The beads conjugated to the targeting peptide of the invention may then be used in assays, such as, for example, to assess binding to endothelial cells. Specifically, human or rabbit aortic endothelial cells in culture were used as a model for vascular endothelium. These cells were routinely proved to be endothelial cells by demonstration of the expression of the known endothelial cell specific markers von Willebrand factor and VE-cadherin (as detailed for example in H.M. Piper, et al. From H.M. Piper, ed., "Cell Culture Techniques in Heart and Vessel Research", Springer-Verlag, Berlin (1990); p. 158-177. VE-cadherin was detected by a modification of the method used by Dejana et. al. (E. Dejana, et al., J. Cell Biol. (1987); 104:1403-1411)). The beads conjugated to the targeting peplide A of the invention were incubated under various conditions with HAEC (human aortic endothelial cell) monolayers in culture and the specifically bound beads quantitated and compared to the underivatized beads or beads derivatized with compounds not known to target endothelial cells.Such as, for example, BSA (bovine serum albumin), the peptide GRGDSP (SEQ ID NO:3), glycine. The results demonstrated that compounds of general formula (IIb) effectively target (bind) derivatized beads to HAEC, while underivatized beads or beads derivatized with BSA, GRGDSP, or glycine do not.

The ultrasound agents of the present invention containing the targeting moiety A were treated in a similar manner. The incubations were also performed under flow conditions to simulate the in vivo environment. These studies demonstrate that the ultrasound agents are also targeted to HAEC by the targeting moiety A, whereas ultrasound agents lacking the targeting moiety do not bind. Further, the binding was shown to extend to aortic endothelial cells from another species,such as rabbits, but not to a non-endothelial human cell line, KB.

In another preferred embodiment of the present invention, the invention includes compounds of the formula A-L-B_2 , where B_2 corresponds to B_{2b} a non-ionic surfactant such as (PVA), polyoxyethylene-polyoxypropylene block copolymers, e.g. Pluronic®, Synperonic®, Poloxamer®, Poloxamine®, or BRIJ®.

These compounds are particularly useful for preparing targeted MRI contrast agents based on lipophilic iron partciclesThe novel compositions of the present invention, and especially the microbubbles and microballoons, are useful as contrast media in diagnostic imaging, and are also suitable as therapeutic agents, in the presence or not of a bioactive agent, as cited above. This may be achieved by administering the compounds of general formula I such that the receptors involved with angiogenesis, such as the NP-1 VEGFreceptor are occupied and unreceptive to endogenous receptor binders such as VEGF.

Another method of therapy is to use the compounds of general formula I as vehicles with which to target bioactive compounds to a desired site.

In one embodiment, the compostions of the invention may be used to deliver one or more bioactive agents. A bioactive agent is a compound that is capable of providing a biological effect, including a therapeutic or cytotoxic effect. In this embodiment the substrate B may be, for example, a known drug delivery vehicle such as, for example, a liposome, a microparticle etc. In a preferred embodiment, the targeting moiety A is a TKPPR multimer such as a TKPPR tetramer.

As bioactive agent is used herein to encompass genetic material, the substrate B may also include a known gene or nucleic acid delivery vehicle (such as, for example, a virus particle, a gene therapy vector, a liposome, a complex of lipids (e.g. cationic lipids) and genetic material, a complex of dextran derivatives and genetic material etc,.)

Additionally, as discussed in more detail herein, A, the targeting peptide of the invention may be conjugated (optionally through a linker) to a bioactive agent-containing gas filled microbubble or microballoon. In this embodiment, the gas filled contrast agent includes the targeting peptide of the invention; thus it is able to target the agent to tumor cells or endothelial cells (and particularly angiogenic endothelial cells). Ultrasound may then be used to rupture the targeted, bioactive agent-

containing ultrasound contrast agent of the invention, thus releasing the bioactive agent.

Interaction of the bioactive agent with the desired target may be whilst still part of the vehicle or upon release from the vehicle which may be by passive or active means. Passive means are those such as diffusion away from the vehicle whilst the vehicle is bound to its target and active means may be those such as insonation of the vehicle to achieve rupture and release of the carried bioactive material.

Any of a variety of bioactive agents may be used in and delivered by the compostions of the invention. By bioactive agent, as used therein, is meant an agent having a beneficial, therapeutic or cytotoxic effect in vivo. As used herein, the term bioactive agent encompasses genetic material and is synonymous with the terms therapeutic, chemotherapeutic, drug, etc. Suitable bioactive agents include, but are not limmied to: antineoplastic agents, such as platinum compounds (e.g., spiroplatin, cisplatin, and carboplatin), methotrexate, adriamycin, mitomycin, ansamitocin, bleomycin, cytosine, arabinoside, arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, melphalan (e.g., PAM, a, L -PAM or phennylalanine mustard), mercaptopurine, mitotane. procarbazine hydrochloride, dactinomycin (actinomycin D), daunorubcin hydrochloride, doxorubicin hydrochloride, taxol, mitomycin, plicamycin (mithramycin), aminoglutethimide, estramustine phosphate sodium, flutamide, leuprolide acetate, megestrol acetate, tamoxifen citrate, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase) *Erwina aparaginase,* etoposide (VP-16), interferon α-2a, interferon α-2b, teniposide (VM-26), vinblastine sulfate (VLB), vincristine sulfate, , bleomycin sulfate, , adriamycin, and arabinosyl; blood products such as parenteral iron, hemin, hematoporphyrins and their derivatives, biological response modifiers such as muramyldipeptide, muramyltripeptide, microbial cell wall components, lymphokines (e.g., bacterial endotoxin such as lipopolysaccharide, macrophage activation factor), sub-units of bacteria (such as Mycobacteria, Corynebacteria), the synthetic dipeptide N-acetyl-muramyl-l-alanyl-l)-isoglutamine; anti-fungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine (5-fc), miconazole, amphotericin B, ricin, and β-lactam antibiotics (e.g., sulfazecin); hormones such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone ,dipropionate, betamethasone, betamethasone acetate and betamethasone sodium phosphate, vetamethsone disodium phosphate, vetemthsone sodium phosphate, cortisoneacetate, dexamethasone, dexamethasone acetate, dexamethasone sodium phosphate, flunisolide, hydrocortisone, hydrocortisone acetate, hydrocortisone cvpionate, hydrocortisone sodium phosphate, hydrocortisone sodium succinate,

methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, paramethasone acetate, prednisolone, prednisotone acetate, prednisolone sodium phosphate, prednisolone tebutate, prednisone, triamcinolone, triamcinolone acetonide, triamcinolone diacetate, triamcinolone hexacetonide and fludrocortisone acetate; vitamins such as cyanocobalamin neinoic acid, retinoids and derivatives such as retinol palmitate, and α-tocpherol; ; enzymes such as manganese super oxide dimsutase or alkaline phosphatase; anti-allergice agents such as amelexanox; anti-coagulation agents] such as phenprocoumon and heparin; circulatory drugs such as propranolol; metabolic potentiators such as glutathione;antituberculars such as para-aminosalicylic acid, isoniazid, capreomycin sulfate cycloscrine, ethambutol hydrochloride ethionamide, pyrazinamide, rifampin, and streptomycin sulfate; antivirals such as acyclovir, amantadine azidothymidine (AZT or Zidovudine), ribavirin and vidarabine monohydrate (adenine arahinoside, ara-A); antianginals such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide dinitrate, nitroglycerin (glyceryl trinitrate) and pentaerythritol tetranitrate; antibiotics, anti-inflammatories such as diflunisal, ibuprofen, indomethacin, meclofenamate, mefenamic acid, naproxen, oxyphenbutazone, phenylbutazone, piroxicam, sulindac, tolmetin, aspirin and salicylates; antiprotozoans such as chloroquine, hydroxychloroquine, metroidazole, quinine and meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, heroin, methadone, morphine and opium; cardiac glycosides such as deslanoside, digitoxin, digoxin, digitalin and digitalis, neuromuscular blockers such as atracutrium mesylate, gallamice triethiodide, hexafluorenium bromide, metocurine iodide, pancuronium bromide, succinylcholine chloride (suxamethonium chloride), tubocurarine chloride and vecuronium bromide; sedatives (hypnotics) such as amobarbital, amobarbital sodium, aprobarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methyprylon, midazolam hydrochloride, paraldehyde, pentobarbital, pentobarbital sodium, phenobarbital sodium, secobarbital sodium, talbutal, temazepam and triazolam; local anesthetics such as bupivacaine hydrochloride, chloroprocaine hydrochloride, etidocaine hydrochloride, lidocaine hydrochloride, mepivacaine hydrochloride, procaine hydrochloride and tetracaine hydrochloride; and general anesthetics such as droperidol, etomidate, fentanyl citrate with droperidol, ketamine hydrochloride, methohexital sodium and thiopental sodium. In certain embodiments, the therapeutic is a monoclonal antibody, such as a monoclonal antibody capable of binding to melanoma antigen.

Other preferred therapeutics include genetic material such as nucleic acids, RNA, and DNA, of either natural or sythetic origin, including recombinant RNA and

DNA and antisense RNA and DNA. Types of genetic material that may be used include, for example, genes carried on expression vectors such as plasmids, phagemids, cosmids, yeast artificial chromosomes (YAC's) and defective or "helper" viruses, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, such as phosphorothioate and phosphorodithioate oligodeoxynucleotides. Additionally, the genetic material may combined, for example, with lipids, proteins or other polymers.

DNA encoding certain proteins may be used in the treatment of many different types of diseases. For example, adenosine deaminase may be provided to treat ADA deficiency; tumor necrosis factor and/or interleukin-2 may be provided to treat advanced cancers, HDL receptor may be provided to treat liver disease; thymidine kinase may be provided to treat ovarian cancer, brain tumors, or HIV infection; HLA-B7 may be provided to treat malignant melanoma interleukin-2 may be provided to treat neuroblastoma, malignant melanoma, or kidney cancer; interleukin-4 may be provided to treat cancer; HIV env may be provided to HIV infection; antisense ras/p53 may be provided to treat lung cancer; and Factor VIII may be provided to treat Hemophilia B. See, for example, *Science* 258, 744-746, incorporated herein by reference.

In accordance with the present invention, there are provided methods of imaging a patient generally, and/or in specifically diagnosing the presence of diseased tissues in a patient. The imaging process of the present invention may be carried out by administering a contrast medium of the invention to a patient, and then scanning the patient using, for example, ultrasound, computed tomography, and/or magnetic resonance imaging or scintigraphy, to obtain visible images of an internal region of a patient and/or of any diseased tissue in that region. By region of a patient, it is meant the whole patient or a particular area or portion of the patient.

Nevertheless, as discussed above, the present invention also provides the possibility to use compositions comprising a monomer, multimer or polymer of timer or polymer of TKPPR or a TKPPR analogue, without the presence of a bioactive agent, as a therapeutic agent useful as an inhibitor of the angiogenesis process.

The administration of the compositions of the present invention is generally parenteral and the amount and the period of time are depending upon a variety of factors including, for example, the volume of the composition to be administered, the weight of the patient, the region of interest etc. Another possible route of administration is the topical application, particularly useful for the skin diseases associated with angiogenesis, as cited above.

The following are embodiments of the invention:

1. A compound of the formula (I)

A-L-B (I)

in which

A is TKPPR or an analogue of TKPPR which specifically binds to an endothelial cell or cells that express markers in common with endothelial cells, with equal or greater avidity as TKPPR, ;

L is a linker;

B is a substrate.

2. A compound according to embodiment 1, wherein B corresponds to B_1, which is a lipid able to bind the linker in a covalent or not covalent manner.

3. A compound according to embodiment 1, wherein B corresponds to B_2, which is a non lipid polymer able to bind the linker in a covalent manner.

4. A compound according to embodiment 2, in which B_1 is a synthetic or naturally-occurring generally amphipathic and biocompatible compound, selected from the group consisting of fatty acids; lysolipids; phospholipids; phosphatidylinositol; sphingolipids; glycolipids; glucolipids; sulfatides; glycosphingolipids; phosphatidic acids; lipids bearing polymers;; lipids bearing sulfonated mono- di-, oligo- or polysaccharides; cholesterol, cholesterol sulfate; cholesterol hemisuccinate; tocopherol hemisuccinate; lipids with ether and ester-linked fatty acids; polymerized lipids; diacetyl phosphate; dicetyl phosphate; stearylamine; cardiolipin; phosholipids with short chain fatty acids of about 6 to about 8 carbons in length; synthetic phospholipids with asymmetric acyl chains; ceramides; non-ionic liposomes;; sterol esters of sugar acids; ; esters of sugars and aliphatic acids; saponins; glycerol dilaurate; glycerol trilaurate; glycerol dipalmitate; glycerol; glycerol esters; ; long chain alcohols; 6-(5-cholesten-3ß-yloxy)-1-thio-ß-D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholesten-3ß-yloxy)hexyl-6-amino-6-deoxy-1-thioß-D-galactopyranoside; 6-(5-cholesten-3ß-yloxy)hexyl-6-amino-6-deoxyl-1-thioß-D-mannopyranoside; 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid; N-succinyl-dioleylphosphatidylethanolamine; 1,2-dioleyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoylglycerophosphoethanolamine; palmitoylhomocysteine, and combinations thereof.

5. A compound according to embodiment 3, in which B_2 is B_{2a} which is a polymer useful for producing microparticles, or B_{2b}, a non-ionic surfactant.

6. A compound according to embodiment 3 selected from the group consisting of PVA, and a polyoxyethylene-polyoxypropylene block copolymer.

7. A compound according to embodiment 4, in which B_{2a} is a bead which is derivatizable and is attached to a detectable label.

8. A compound according to embodiment 7, in which the detectable label is a fluorescent or radioactive marker.

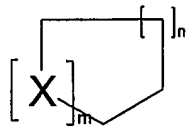
9. A compound according to embodiments 1 to 8, in which L is a bond or is derived from :

an alkyl chain C_1-C_{6000}, linear or branched, saturated or unsaturated, optionally interrupted or substituted by one or more groups such as: O, S, NR, OR, SR, COR, COOH, COOR, CONHR, CSNHR, C=O, S=O, $S(=O)_2$, $P=O(O)_2OR$, $P(O)_2(OR)_2$, halogens, or phenyl groups, optionally substituted by one or more -NHR, -OR, -SR, -COR, -CONHR, -N-C=S, -N-C=O, halogens, in which

R is H or an alkyl group C_1-C_4, linear or branched, optionally substituted by one or more –OH;

such a chain can be interrupted or substituted by one or more cyclic groups C_3-C_9, saturated or unsaturated, optionally interrupted by one or more O, S or NR; by one or more groups such as: -NHR, -OR, -SR, -COR, -CONHR, or a phenyl group optionally substituted by one or more -NHR, -OR, -SR, -COR, -CONHR, -N-C=S, -N-C=O, halogens.

10. A compound according to embodiment 9, in which the cyclic groups present in L are saturated or unsaturated, and correspond to the following general formula (III)



(III)

in which

n can range from 0 to 4;

m can range from 0 to 2;

X can be NH, NR, O, S or SR.

11. A compound according to embodiment 10, in which the linker L is an oligopeptide constituted from 1 to 100 of natural or synthetic amino acids.

12. A compound according to embodiment 11, in which the aminoacids are selected in the group from glycine, glutamic acid, aspartic acid, γ-amino-butyric acid, trans-4-aminomethyl-cyclohexane carboxylic acid.

13. A compound according to embodiment 10, in which the L precursor corresponds to difunctional PEG(polyethyleneglycol) derivatives.

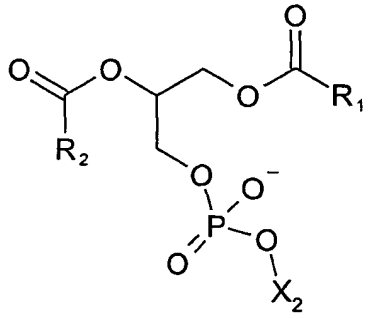
14. A compound according to embodiment 10, in which L is selected in a group consisting of: glutaric acid, succinic acid, malonic acid, oxalic acid, PEG derivatized with two CH_2CO groups.

15. A compound of the formula (IIa), according to embodiment 2

$$A\text{-}L\text{-}B_{1a} \quad (IIa)$$

in which

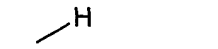
B_{1a} is a phospholipid moiety of the formula (II),



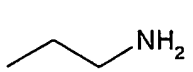
M (II)

and

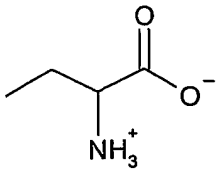
M	is an alkaline or alkaline- earth metal cation;
R_1 and R_2	independently, correspond to a linear long chain C_{12}-C_{20}; saturated or unsaturated, optionally interrupted by C=O, or O;
X_2	is selected in a group consisting of



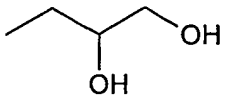
phosphatidic acid



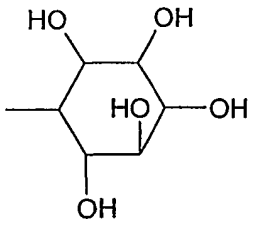
ethanolamine



serine



glycerol



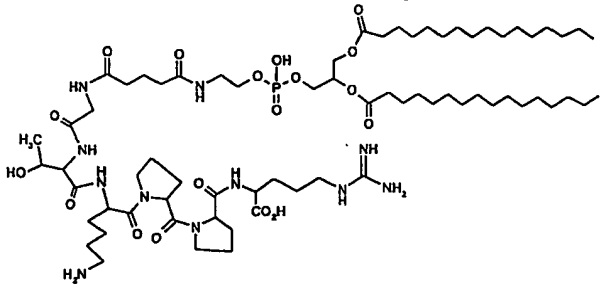
inositol

16. A compound according to embodiment 15, in which R_1 and R_2 are independently a saturated linear long chain C_{12}-C_{20}.

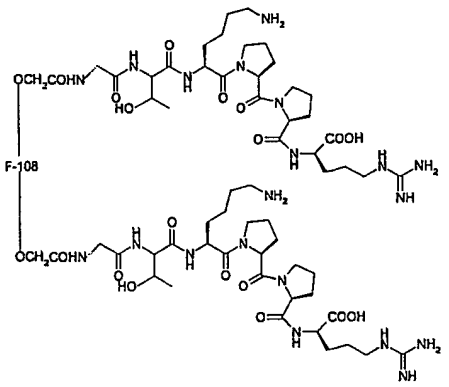
17. A compound according to embodiment 16, in which the phospholipids of formula (II) are selected in the group from: dimyristoylphosphatidylethanolamine, dipalmitoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, diarachidoylphosphatidylethanolamine, dioleylphosphatidylethanolamine, dilinoleylphosphatidylethanolamine, fluorinated analogues of any of the foregoing, and mixtures of any of the foregoing.

18. A compound according to embodiment 17, in which the phospholipid of formula (II) is dipalmitoylphosphatidylethanolamine.

19. A compound selected in the group consisting from:



and



20. A process for preparing a compound of embodiment 1 comprising the following steps:

obtaining TKPPR or an analogue thereof;

conjugating TKPPR with the linker to give a compound of formula (IIb)

A-L (IIb)

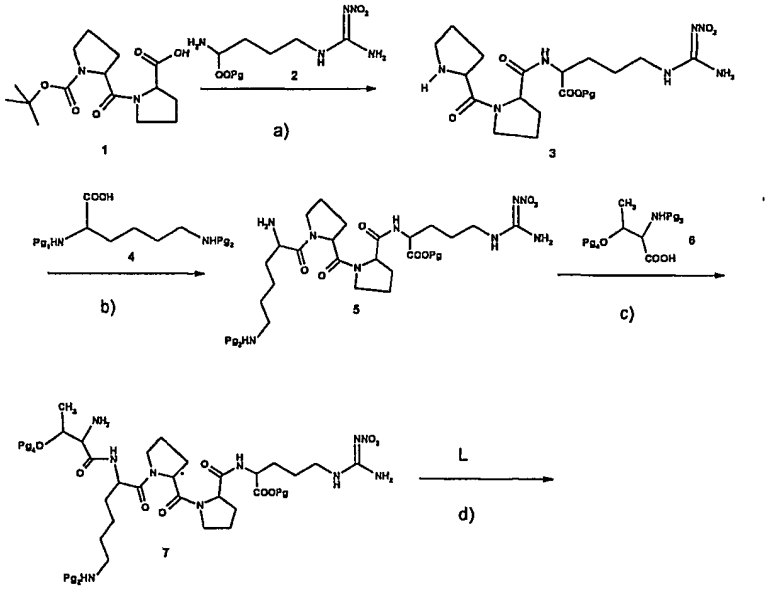
; and

forming a covalent or non-covalent bond between a compound of formula (IIb) and the substrate B or

forming a covalent bond between the substrate B and the linker to form a conjugate B-L, and

conjugating of the conjugate B-L with TKPPR or an analogue thereof.

21. A process according to embodiment 20, in which the compounds of formula (IIb) are prepared as illustrated in the following Scheme



(Pg = protecting group)

in which

the steps a), b), and c) are all condensation reactions performed under basic conditions, and step d) is a condensation in basic conditions with the linker.

22. A composition for targeting endothelial cells or cells that express markers in common with endothelial cells, of humans and animals, in vivo or in vitro, and/or for administration of at least one bioactive agent, comprising at least one of the compounds of embodiment 1 with an optional detectable moiety.

23. A composition, according to embodiment 22, comprising an ultrasound detectable moiety and at least one of the compounds of formula (Ia) or (IIa).

24. A ccomposition, according to embodiment 22, further comprising at least one bioactive agent incorporated in the detectable moiety.

25. A composition, according to embodiment 23, comprising a compound of formula (Ib), for targeting said ultrasound detectable moiety .

26. A method of imaging an angiogenic site in an human or animal comprising administering to said animal a composition comprising a detectable moiety and a compound of formula (Ia) or (IIa) and detecting said moiety at an angiogenic site.

27. A method of staging a tumor in an animal comprising administering a composition comprising a detectable moiety and a compound of formula (Ia) or (IIa) to said animal and detecting said moiety in said animal.

28. A method of ultrasound imaging comprising administering an ultrasound contrast media composition comprising a compound of formula (Ia) or (IIa) to said animal and imaging said contrast agent in said animal.

29. A method of screening at least one agent for the specificity of said agent to target endothelial cells or cells that express markers in common with endothelial cells, of an animal, comprising administering to said animal or contacting said cells in vitro with a composition comprising a compound of formula (Ib) and detecting said specificity.

30. A method of screening at least one targeted ultrasound contrast media, according to embodiment 29, comprising administering or contacting a compound of formula (Ib).

31. A method for the therapeutic delivery in vivo of a bioactive agent to a patient suffering from effects associated with angiogenesis disorders comprising administering a therapeutically effective amount of a composition comprising a compound of formula (Ia) or (IIa).

32. A method of treating an individual experiencing an effect of an angiogenesis disorder comprising administering a therapeutically effective amount of a composition comprising a compound of formula (Ia) or (IIa).

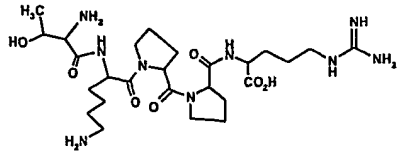
The invention is further demonstrated in the following examples. The examples are for purposes of illustration and are not intended to limit the scope of the present invention.

The disclosure of all of the above-described references, patents and patent applications are incorporated herein by reference in their entirety.

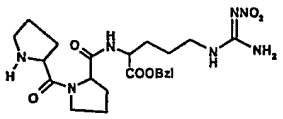
EXAMPLES

The stereochemistry of the chemical bonds in the drawings of the Examples will be omitted because the amino acids all have the natural configuration S at the chiral center and there is always retention of configuration in the exemplified reactions.

Example 1
Preparation of TKPPR-OH



A) Preparation of Pro-Pro-Arg(NO_2)Obzl



To a solution of Boc-Pro-Pro-OH (commercially available) (3.2 g, 10.25 mmol) in methylene chloride (100 mL) was added Arg(NO_2)Obzl.PTSA salt (commercially available) (6.54 g, 10 mmol) and the mixture was stirred for 5 min. This mixture was cooled to 5°C and HATU ([O-(7-azabenzotriazol-1-yl)1,1,3,3,-tetramethyluronium hexafluorophosphate], (commercially available), (3.9 g, 10.25 mmol) was added in one lot followed by diisopropylethylamine (6.5 g, 50 mmol). After stirring the reaction mixture for 12 h at room temperature, the solvents were removed in vacuo, the residue dissolved in ethyl acetate and washed with saturated sodium bicarbonate, sodium bisulphate and finally with water. The organic layer was dried and solvent

removed to afford the coupled product. This was purified by column chromatography over silica gel using 5 % methanol in ethyl acetate as the eluent. Fractions containing the pure material were combined and solvent removed to obtain the pure product. To a solution of this protected tripeptide (5.42 g, 9 mmol) in methylene chloride (12 mL) was added trifluoro acetic acid (TFA)(12 mL) and the mixture was stirred for 1 hr at room temperature. TFA and methylene chloride were removed in vacuo and the residue stirred with anhydrous ether for 15 min. The precipitated solid was collected and dried to afford 5.2 g of the title compound, as the TFA salt.

Yield: 95 %

HPLCPurity: 100%

Retention Time: 9.8 min

Column: YMC, C-18 (4.6 x 250 mm)

Solvent: Water-Acetonitrile, both containing 0.1% TFA

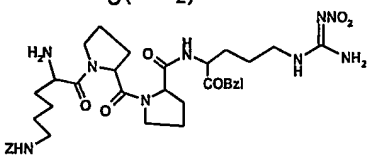
Elution condition: Initial, 20 % acetonitrile, linear gradient to 100 % acetonitrile in 30 min;

Flow rate: 1.0 mL/min

Detection: UV 254 nm.

^{1}H-NMR, and HRMS spectra are consistent with the structure

B) Preparation of Lys(Z)-Pro-Pro-Arg(NO_2)Obzl

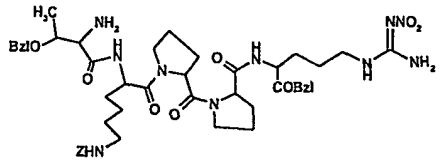


To a solution of Z protected lysine (commercially available) (3.05 g, 8.02 mmol) in methylene chloride (60 mL) was added the TFA salt of Pro-Pro-Arg(NO_2)Obzl (4.93 g, 8 mmol) and the mixture was stirred for 5 min. This mixture was cooled to 5°C and HATU (3.05 g, 8.02 mmol) was added in one lot followed by diisopropylethylamine (4.16 g, 32 mmol). After stirring the reaction mixture for 6 h, the solvents were removed in vacuo, the residue dissolved in ethyl acetate and washed with saturated sodium bicarbonate, sodium bisulphate and finally with water. The organic layer was dried and solvent removed to afford the coupled product. This was purified by column chromatography over silica gel (150 g) using 5 % methanol in ethyl acetate as the eluent. Fractions containing the pure material were combined and solvent removed to obtain the pure product. A solution of this protected tetra peptide (6.0 g, 7 mmol) in methylene chloride (15 mL) was added TFA (15 mL) and the mixture stirred for 1 hr at room temperature. TFA and methylene chloride were removed in vacuo and the residue stirred with anhydrous ether for 15 min. The precipitated solid was collected and dried to afford 5.8 g of the title compound, as the TFA salt.

Yield:	95%
HPLC:	95.7%
Retention Time:	14.02 min.
Column:	YMC, C-18 (4.6 x 250 mm)
Solvent:	Water-Acetonitrile, both containing 0.1% TFA
Elution condition:	Initial 20 % acetonitrile, linear gradient to 100% acetonitrile in 30 min
Flow rate:	1.0 mL/min
Detection:	UV 254 nm.

^{1}H-NMR, and HRMS spectra are consistent with the structure

C) Preparation of Thr(Obzl)-Lys(Z)-Pro-Pro-Arg(NO_2)Obzl



To a solution of Boc-threonine benzyl ether, (commercially availble) (1.96 g, 6.3 mmol) in methylene chloride (50 mL) was added TFA salt of Lys(Z)-Pro-Pro-Arg(NO_2)Obzl (5.25 g, 6 mmol) and the mixture was stirred for 5 min. This mixture was cooled to 5°C and HATU (2.41 g, 6.3 mmol) was added in one lot followed by diisopropylethylamine (3.35 g, 25 mmol). After stirring the reaction mixture for 4 h at room temperature, the solvents were removed in vacuo, the residue dissolved in ethyl acetate and washed with saturated sodium bicarbonate, sodium bisulphate and finally with water. The organic layer was dried and solvent removed to afford the coupled product. This was purified by column chromatography over silica gel (150 g) using 5% methanol in ethyl acetate as the eluent. Fractions containing the pure material were combined and solvent removed to obtain the pure product (5.08 g, yield 91%). A solution of this protected penta-peptide (2.1 g, 2 mmol) in methylene chloride (4 mL) was added TFA (4 mL) and the mixture stirred for 1 hr at room temperature. TFA and methylene chloride were removed in vacuo and the residue stirred with anhydrous ether for 15 min. The precipitated solid was collected and dried to afford 2.1 g of the title compound as the TFA salt.

Yield:	98 %
HPLC:	98.3%
Retention Time:	16.12 min
Column:	YMC, C-18 (4.6 x 250 mm)
Solvent:	Water-Acetonitrile, both containing 0.1% TFA
Elution condition:	Initial, 20 % acetonitrile, linear gradient to 100 % acetonitrile in 30 min

Flow rate: 1.0 mL/min.

Detection: UV 254 nm

^{1}H-NMR, and HRMS spectra are consistent with the structure.

D) Preparation of TKPPR-OH

To a solution of the above compound (300 mg, 0.28 mmol) in methanol (30 mL) and acetic acid (3.0 mL) was added $Pd(OH)_2$ (Degussa type, 100 mg) and the mixture was hydrogenated at 50 psi for 48 hr. The catalyst was filtered off and the solvents were removed to afford the crude product. This was triturated with anhydrous ether to obtain the product as a white powder. This crude product was further purified by preparative HPLC on a C-18 column using a linear gradient of 0-30% acetonitrile in 60 min. Fractions containing pure compound were combined and lyophilized to afford 210 mg of the pure TKPPR-OH.

Yield: 84%).

Retention Time: 13.40 min.

Column: YMC, C-18 (4.6 x 250 mm)

Solvent: Water-Acetonitrile, both containing 0.1% TFA

Elution condition: Initial, 0% acetonitrile, linear gradient to 30% acetonitrile in 30 min

Flow rate: 1.0 mL/min.

Detection: UV 220 nm.

Elemental Analysis:

	C	H	N
Calcd.	40.13	5.47	13.16
Found	40.55	5.55	12.79

^{1}H-NMR, and HRMS spectra are consistent with the structure and with the literature data

Example 2

Endothelial cell binding of TKPPR-conjugated fluorescent beads to HAEC

A) Cell Culture

Human aortic endothelial cells (HAEC) from Biowhittaker were grown as monolayers in EGM-MV medium from Biowhittaker according to the supplier's instructions.

Briefly, a frozen cryovial of cells (500,000 cells in about 1 mL) was thawed for 2-3 minutes in a 37°C water bath and cells were seeded into a T-75 flask coated with collagen I (commercially available) containing 15 mL EGM-MV of medium pre-equilibrated with 5% CO_2 atmosphere. Cells were incubated in a standard tissue

culture incubator at 37°C. HAEC were subcultured for up to 3 additional passages, using the following protocol:

- Culture medium from confluent T75 flasks of HAEC (6-8 days after seeding) was removed by aspiration, and cells were washed with Dulbecco's phosphate-buffered saline without Mg^{++} or Ca^{++} (commercially available).
- They were then trypsinized as recommended by Biowhittaker.
- The resulting cell suspension was pelleted by centrifugation. The cell concentration was determined, and a volume of the resuspended cells containing 450,000 cells was added to a collagen I-coated T-75 flask (seeding density = 6,000 cells/cm^2) and fresh culture medium was added to bring the final volume of the flask to 15 mL. Flasks were incubated at 37°C in a standard tissue culture incubator in 5% CO_2 atmosphere, with loosened caps to allow gas exchange. The next day, medium was aspirated to remove non-adherent cells, and replaced with fresh medium. Thereafter, medium was replaced every 2-3 days.
- For binding studies and characterization, pelleted cells were diluted to a concentration of 16,560 cells per mL, and 0.5 mL was seeded into each well of an 8-well chamber slide (Collagen I-coated, Becton Dickinson) to generate a seeding density of 12,000 cells/cm^2. Cells seeded into chamber slides were used for assays after 7 to 10 days, and were not further propagated.

B) Immunofluorescent demonstration of endothelial cell markers

Post-confluent (8-14 days in culture) HAEC that had been fixed in ice-cold methanol 5 min and air-dried 15 min were stained for von Willebrand Factor. Post-confluent HAEC were fixed in 4% paraformaldehyde and 2% sucrose in D-PBS for 5 min, then stained for VE-cadherin.

Staining was evaluated using an Olympus IMT-2 microscope equipped with a mercury lamp (Chiu Technical Corp, Model M-100) for fluorescence detection and employing either a fluorescein filter set, a Texas Red filter set, or a dual fluorescein/Texas Red filter set.

C) Preparation of Peptide-conjugated Microspheres

TKPPR (see preparation described in the Example 1) was attached to red fluorescent carboxylate-modified FluoSpheres™ (Molecular Probes), which are 2.0 μm microspheres provided at 3.9 x 10^9 particles/mL of distilled water. 1.0 mg TKPPR was combined with 0.5 mLof 50 mM MES buffer (2-[N-Morpholino]ethanesulfonic acid, (commercially available), pH 6.0 and 0.2 mL FluoSpheres (7.8 x 10^8 spheres) in a 1.5 mL Eppendorf snap top, polypropylene centrifuge tube and rotated for 30 min at room temperature (RT). Then 2.8 mg EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, (commercially available) in 0.025 mL MES was added and the tube was rotated for 2 h at RT. Following the addition of

0.005 mL 1 N NaOH and 5.7 mg glycine in 0.025 mL MES, the tube was rotated for 30 min more at RT. The beads were then washed by employing three cycles of centrifugation at 14,000 rpm (20,800 x g) in an Eppendorf 5417R centrifuge for 15 min, discarding the resulting supernatant, and resuspending in D-PBS. Storage was at 4°C in 0.4 mL DPBS containing 0.05% NaN_3 (final bead concentration was about 1.95 x 10^9/mL). Conjugation of glycine, GRGDSP or BSA to the microspheres were carried identically except that BSA was added at 3.0 mg to 0.5 mL buffer.

D) TKPPR-Conjugated Bead Binding Experiments

Experiment 1.

Red fluorescent microspheres derivatized with TKPPR, GRGDSP, or BSA (as described above) were diluted at 10 µL / mL EBM medium (Biowhittaker) supplemented with 0.1% (w/v) BSA (Sigma) and 20 µL / mL aprotinin (Sigma). Final bead concentration was 1.95 x 10^7/mL. Unconjugated microspheres were diluted at 5 µL / mL EBM/BSA buffer to give the same microsphere concentration (1.95 x 10^7/mL) achieved with 10 µL / mL of the conjugated preparations. Before starting the assay, bead suspensions were disaggregated in a sonicating bath for 15 min. The wells of an 8-well chamber slide of confluent HAEC were drained of medium and rinsed with 0.5 mL per well of EBM/BSA buffer (without microspheres). To one well each, 250 µL of the following bead solutions (containing 4.9 x 10^6 beads) were added: TKPPR-conjugated, BSA-conjugated, and unconjugated. The slide was incubated 30 min on an orbital shaker, drained, then washed once with 0.5 mL/well EBM/BSA buffer, and twice with 0.5 mL/well D-PBS containing 2 mM $MgCl_2$. Methanol was applied to the outside of the well separating scaffold to loosen the adhesive, then the scaffold was pulled off and the slide mounted with Gel/Mount (Biomeda) or Vectashield aqueous mounting medium.

Bead binding and localization was assessed at 200X magnification using the same microscopy equipment as described above. Digital images were collected of three different random fields in each well. The images were segregated into separate red or green channels in Adobe PhotoShop (image processing software, version 5.0), flattened (layer information removed), and saved as individual TIFF files. Micrografx Picture Publisher (version 7) was then used to enhance contrast by 100%. Finally, the processed images were inverted into black on a white background using Scion Image software (version beta 3b) and the integrated density was measured with the whole field selected.

Results

The integrated density is in arbitrary units selected by the image analysis software.

Bead Type	Integrated density of bound beads

TKPPR	229.97
GRGDSP	28.59
BSA	1.61
Unconjugated	8.11

It is evident that the TKPPR sequence specifically targets the beads to the endothelial cells.

Experiment 2.

Details were as in experiment 1, except that the microsphere concentration was decreased to 4.9×10^6 / mL (lowering to 1.2×10^6 the number of microspheres added per well) for each of the bead types, and the incubation buffer was changed to D-PBS containing 0.1% BSA and 10 μL / mL Sigma protease inhibitor cocktail (P-8340). The incubation time was decreased to 15 min on an orbital shaker, and the washes (3) were carried out using D-PBS containing 0.1% Tween 20 (Sigma). Also, in this experiment, cold methanol was added directly to the wells to fix the cells as well as loosen the scaffold. After scaffold removal, a final D-PBS rinse was added to the protocol before coverslipping. TKPPR-bead binding was quantitated in three fields, the other bead types were quantitated in one field only. Bound beads were quantitated by manual counting.

Results

Bead Type	Number of bound beads
TKPPR	74, 103, 72 (ave. 83)
GRGDSP	2
BSA	1
Unconjugated	5

It is evident that the TKPPR sequence specifically targets the beads to the endothelial cells.

E) Free TKPPR Peptide Inhibition of TKPPR-Conjugated Bead Binding to HAEC

HAEC were seeded into each well of an 8-well chamber slide (Collagen I Cellware, Becton Dickinson) and allowed to achieve confluence at 37°C. Solutions containing both TKPPR-beads (final concentration 4.9×10^6/mL) and green control beads (final concentration 4.9×10^6/mL) were prepared in D-PBS containing protease inhibitor cocktail (final dilution 1:50) and 0, 10, 25, 50, 100, 200, or 500 μM free TKPPR peptide. 0.25 mL of each microsphere solution was added to a well in the drained chamber slide and incubated at RT for 15 min. Two wells received the bead solution (mixed unconjugated and TKPPR beads as indicated above) lacking free competing TKPPR peptide. The slide was then washed three times with 0.5 mL D-PBS containing 0.1% Tween 20, fixed with methanol for 1 min and mounted. The three images for each level of TKPPR competition and the six images of the control

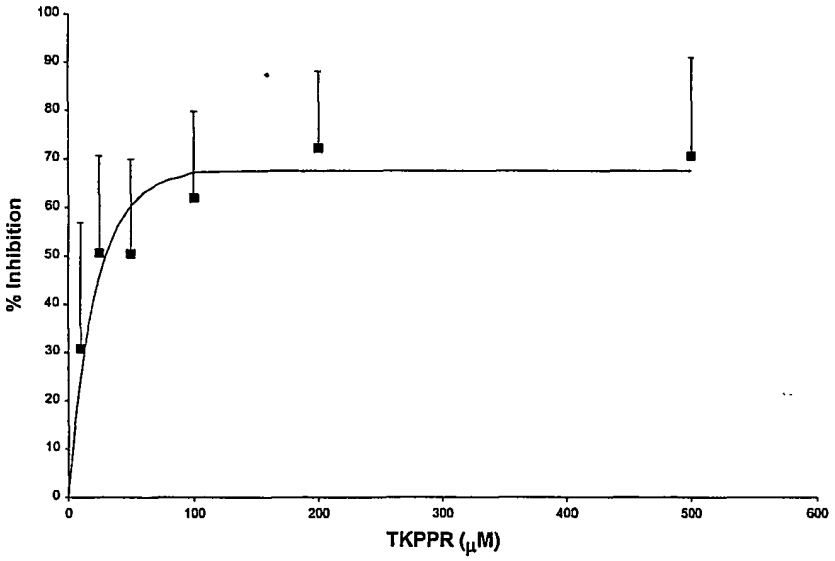
were averaged and the % inhibition of binding was calculated using the following equation:

% Inhibition = 100 x (Control Density – Competition Density) / Control Density

A total of eleven 8-well slides were evaluated for competitive TKPPR-bead binding to HAEC on 7 different days.

Free TKPPR [μM]	Average (n=11) % Inhibition	SD	%CV
10	30.8	26.1	84.7
25	50.7	20.0	39.4
50	50.5	19.5	38.6
100	62.0	17.8	28.7
200	72.2	15.9	22.0
500	70.6	20.3	28.8

The % inhibition data clearly show that TKPPR-beads bind to HAEC in a competitive manner, further illustrated in the chart below. The equation $y=A*(1-e^{-ax})$ was fit to the % Inhibition data to develop a curve which best fits the data using CONSAM software.



These results demonstrate that the TKPPR peptide sequence can direct specific binding of fluorescent microspheres to HAEC. This binding can be competed with free peptide.

Example 3

Evaluation of TKPPR-bead binding to endothelial cells under flow

A) Collagen-coating of Glass Coverslips